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Evaluation of methods for inactivating porcine epidemic diarrhea virus (PEDV) in livestock trailers

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Evaluation of methods for inactivating porcine epidemic diarrhea virus (PEDV) in livestock trailers

by

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NOMENCLATURE

CCV	Canine Coronavirus
Ct	Cycle Threshold
HCoV-229E	Human Coronavirus-229E
HCoV-OC43	Human Coronavirus-OC43
IHC	Immunohistochemical
MERS-CoV	Middle Eastern Respiratory Syndrome Coronavirus
MHV	Mouse Hepatitis Virus
MPN	Most Probable Number
PED	Porcine Epidemic Diarrhea
PEDV	Porcine Epidemic Diarrhea Virus
PFU	Plaque Forming Units
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
RH	Relative Humidity
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SARS-CoV	Severe Acute Respiratory Syndrome-associated Coronavirus
ssRNA	Single-Stranded Ribonucleic Acid
TADD	Thermo-Assisted Drying and Decontamination
TCID50	50% Tissue Culture Infective Dose
TGEV	Transmissible Gastroenteritis Virus

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ABSTRACT

Since the introduction and detection of PEDV in the US in the spring of 2013, the swine industry has witnessed incredibly rapid spread of the virus to sow herds and growing pigs across the geographical US. The swine industry is an "industry on wheels," with transport of large numbers of swine across the country every day. It is not surprising then, to discover that contaminated livestock trailers have played a role in the movement of this virus. We cannot quickly change the mobile nature of swine production, but we can identify and implement effective decontamination procedures for contaminated livestock trailers.

The body of literature reviewing inactivation of PEDV is very small, but there are a great number of studies that describe inactivation of other, structurally similar, coronaviruses. These studies indicate that several of the typical methods employed for livestock trailer decontamination, e.g. chemical disinfection and exposure to high temperatures, as well as other environmental conditions such as alkaline pH and high relative humidity, or prolonged time at moderate temperatures were all capable of effectively inactivating coronaviruses. Additionally, it was evident that the presence of proteins around the virus (as might be present in feces) decreased the effectiveness of both heat and disinfectant at virus inactivation, and that chemical disinfectants are more effective when applied at higher temperatures.

To directly investigate the ability of high temperatures to inactivate PEDV in the context of a livestock trailer and TADD facility, original research was conducted to evaluate multiple temperatures applied to PEDV-contaminated feces on aluminum trays. PEDV inactivation was determined via swine bioassay. In this study only 71°C for 10 minutes and

20°C for 7 days were capable of preventing PEDV-infection in 100% (4/4, each) of the swine bioassays.

A second study evaluated the ability to eliminate and inactivate PEDV through the use of high pressure washing with detergent, application of a combination gluteraldehyde and quaternary ammonium chloride disinfectant, Synergize, with and without heating at various temperatures. Swine bioassay again was used to determine infectivity. In this study, all treatments groups resulted in inactivation of PEDV to a level of preventing infection of 100% (4/4) of swine bioassays.

The combination of these studies and review suggest that a sound trailer decontamination program should focus on removal of fecal and organic material, followed by chemical disinfection, and finally heating to high temperature. Each of these steps when performed in combination will be more effective than each by itself, i.e. “the whole is greater than the sum of its parts.” These suggestions represent procedures that can be easily implemented in today’s swine industry. Although trailers and decontamination facilities are in short supply, the knowledge and technology to accomplish these processes is widespread and should be more widely adopted to prevent the continued spread of PEDV.

CHAPTER 1

GENERAL INTRODUCTION

Thesis introduction

Since the identification of porcine epidemic diarrhea virus (PEDV) in the United States in May 2013, the swine industry has seen rapid spread of the virus to 30 states and well over 1,000 premises (USDA, 2014 and USDA, 2015). This rapid and widespread dissemination of the virus is due in part to livestock trailers that become contaminated at one premise before visiting other premises (Lowe et al., 2014). If we wish to slow and stop the spread of PEDV through the US swine herd, we must first stop the movement of the virus between premises on these contaminated livestock trailers. One means to stop this movement is to identify effective means of decontaminating trailers once they have visited a PEDV-positive premise.

As a group, US pork producers have already identified and partially employed methods for decontaminating trailers that are potentially contaminated with other swine pathogens such as PRRSV. However, little to no information is known regarding methods for decontaminating livestock trailers that have been contaminated with PEDV. It is possible that our current decontamination procedures would be sufficient, but since so little research has been conducted in this area – especially relating specifically to livestock trailers – it is impossible to know.

This thesis will serve as a complete summary of what is already known about PEDV and other coronavirus inactivation properties as they might relate to trailer decontamination. It will also serve to explore and summarize specific trailer decontamination procedures for

the elimination and inactivation of PEDV and their ability to prevent infection of live pigs. The goal of this thesis will be to elucidate PEDV inactivation properties and to identify practical and effective means to decontaminate livestock trailers contaminated with PEDV so the continued spread of this virus across the US can be stopped.

Thesis organization

This thesis is composed of five chapters. Chapter 1 is a brief introduction, summary of the thesis organization and objectives. Chapter 2 is titled "A review of methods sufficient to inactivate coronaviruses," and is a literature review relating to the different conditions under which different coronaviruses are inactivated, including temperature, time, chemical disinfectants, pH, and relative humidity. The information contained in this chapter served as a guide for the studies described in chapters 3 and 4. Chapter 3 "Evaluation of time and temperature sufficient to inactivate porcine epidemic diarrhea virus in swine feces on metal surfaces," was published in the March 2015 issue of the *Journal of Swine Health and Production*. Chapter 4 is the manuscript for a study titled "Evaluation of combinations of time and temperature sufficient to inactivate porcine epidemic diarrhea virus (PEDV) on a metal surface following a wash with detergent and disinfection with a quaternary ammonium chloride and gluteraldehyde disinfectant." This manuscript has not been published in its current form, but will be submitted to the *American Journal of Veterinary Research* for publication. Chapter 5 includes a summary and conclusion with remarks on proper trailer decontamination procedures for the elimination and inactivation of PEDV.

Thesis objectives

The objective of Chapter 2 is to identify what is already known about the properties of PEDV and other coronavirus survival/inactivation characteristics under different chemical

and physical conditions such as temperature exposures, differing pH conditions, exposure to chemical disinfectants, and variations in relative humidity (RH). Specifically, the goal was to identify and describe those conditions that could be used to eliminate and inactivate PEDV from livestock trailers. Chapter 3 focused specifically on the application of heat to inactivate PEDV in feces. The objective of Chapter 3 is to determine temperature and time applications that are sufficient to inactivate PEDV on a commercial livestock trailer, and practical within the constraints of current thermo-assisted drying and decontamination (TADD) capabilities in the swine industry. Chapter 4 builds on what was learned in Chapter 3. The objectives of Chapter 4 are to investigate a range of industry-relevant time and temperature combinations following wash and disinfection steps to determine if they are sufficient to inactivate PEDV in swine feces in livestock trailers. This chapter evaluates entire decontamination protocols as would be typical in the swine industry and builds on the temperature ranges identified in Chapter 3.

CHAPTER 2

A REVIEW OF METHODS SUFFICIENT TO INACTIVATE CORONAVIRUSES

Introduction

Until recently, porcine epidemic diarrhea virus (PEDV), an alphacoronavirus and member of the family *Coronaviridae*, was considered to be absent from the western hemisphere (Pospischil et al., 2002; Stevenson et al., 2013). In May of 2013, PEDV was identified in swine in the United States for the first time. The virus causes severe diarrhea in sows and piglets due to enteric villous atrophy. Mortality approaches 100% in piglets (Stevenson et al., 2013). Outbreaks of porcine epidemic diarrhea (PED) continue to occur in the United States, with nearly 7,000 PEDV-positive accessions reported from 30 states as of June 2014 (USDA, 2014), and 1,123 PEDV-positive premises confirmed since that data began being collected in June 2014 (USDA, 2015). Genetic analysis of PEDV isolates from affected farms in the United States found the virus to be 99% genetically similar to isolates from China (Chen et al., 2014; Huang et al., 2013; Hoang et al., 2013), suggesting an Asian source.

Although the original mode of entry of PEDV into the United States remains unknown, contaminated livestock trailers certainly represent a significant risk for movement of the virus between and within herds (Lowe et al., 2014). Historically, the disease risk posed by contaminated trailers has been effectively mitigated in some cases with the use of trailer washing, disinfection protocols, and thermo-assisted drying and decontamination (TADD) systems (Dee et al., 2005). Similar trailer decontamination practices will be critical in

preventing the continued spread of PEDV, but effective parameters for decontamination of trailers contaminated with PEDV have not yet been defined.

In order to make the best recommendations to producers and transporters, the stability of PEDV must be understood and parameters for the inactivation of PEDV must be defined. However, very little research has been conducted at this time into the inactivation properties of PEDV. This review is an attempt to summarize what is known about the stability of coronaviruses and means for their effective inactivation as a surrogate for PEDV.

Materials and methods

Definition of the review question

What are the physical, chemical, and other parameters required for inactivation of coronaviruses that are relevant to the conditions found in or that can be applied to livestock trailers?

Specifically, the author is interested in parameters for inactivation by heating, application of a chemical disinfectant common to the swine industry, or changing pH; and how these parameters might vary under different conditions of time, relative humidity, or in the presence of another material (feces, water, etc.).

Scope (PICO)

Participants

Participants include any virus of the family *Coronaviridae* and genera *Alphacoronavirus* or *Betacoronavirus*, regardless of host species or nature of disease caused by the virus. All coronaviruses are enveloped, single-stranded RNA (ssRNA) viruses. These physical properties are expected to cause them to be susceptible to similar methods and parameters for inactivation.

Intervention

Application of several means for inactivation, including:

- Heat
- Chemical disinfection
- Changing the pH
- Changes in relative humidity
- Time-dependent virus inactivation

Comparisons

Studies comparing one or more intervention (as described above) to no intervention, under the same conditions will be included.

Outcomes

- Reduction in 50% tissue culture infective dose (TCID50)
- Reduction in plaque forming units (PFU)
- Changes in cycles to threshold (Ct)
- Change in genomic copies
- Bioassay outcomes
- Other objective quantifiable measures of virus reduction or inactivation

Indexes Searched

The National Library of Medicine (PubMed) was searched to identify articles that meet the requirements set forth above. Additionally the Swine Information Library maintained by the American Association of Swine Veterinarians was searched to identify peer-reviewed articles published in the Journal of Swine Health and Production that relate specifically to swine but are not indexed in PubMed.

Search Terms

The search terms used for both PubMed and the Swine Information Library were: (Thermal* OR Heat* OR Temperature* OR humidity OR chemical* OR disinfect* OR time OR pH) AND (Coronavir* OR sars* OR pedv OR porcine epidemic diarrhea virus OR transmissible gastroenteritis OR tgev) AND (inactivat* OR surviv* OR destr*)

Search of both databases was performed on March 21, 2015.

Screening Criteria

1. Studies must have evaluated a method for inactivating one or more viruses belonging to the family *Coronaviridae* and genera *Alphacoronavirus* or *Betacoronavirus*.
2. Studies must be full-length, peer-reviewed articles.
3. Outcomes described in the study must be measurable and expressed objectively.
    a. For example, reduction in TCID50
4. Studies from any year or country will be accepted, but must be published in English.

Results

The search terms returned 892 results from PubMed. Of the 892 PubMed results, only 28 were related to inactivation of alpha- and betacoronaviruses. Of those, three were not full-length articles, so only 25 studies were included for the review.

The same search terms returned 42 results from the Swine Information Library. Of those 42 results, several were duplicate listings and only one article related to inactivation of a coronavirus. This study was further excluded from the review because it is included in its entirety in a later chapter of this thesis.

Many of the studies summarized in this review included the evaluation of multiple viruses, some of which were not coronaviruses. The data regarding these viruses was

excluded from this review. Additionally, several studies evaluated means of inactivation, such as gel-based hand disinfectants or household aerosol spray products, which could not possibly be applied to livestock environments efficiently. Because this review is meant to identify and summarize inactivation methods that might be representative of swine facilities, data regarding these products was also excluded from the review.

Description of coronaviruses

The studies summarized here involved work with eight different coronaviruses – four alphacoronaviruses and four betacoronaviruses – with a wide host range including swine, humans, dogs, and mice. The viruses included are porcine epidemic diarrhea virus (PEDV), transmissible gastroenteritis virus (TGEV), canine coronavirus (CCV), human coronavirus 229E (HCoV-229E), human coronavirus OC43 (HCoV-OC43), severe acute respiratory syndrome associated coronavirus (SARS-CoV), middle eastern respiratory syndrome associated coronavirus (MERS-CoV), and mouse hepatitis virus (MHV).

Porcine epidemic diarrhea virus is an alphacoronavirus that causes enteritis, diarrhea, and vomiting in swine. Disease is limited in adults and immune populations, but can approach 100% mortality in young suckling piglets. Distribution of the virus is worldwide today.

Transmissible gastroenteritis virus is an alphacoronavirus that causes disease and clinical signs nearly identical to PEDV in swine. Distribution is worldwide.

Canine coronavirus is an alphacoronavirus that causes a mild enteritis in puppies. Recovery is usually swift unless a mixed viral infection (parvovirus, others) occurs. Distribution is worldwide.

Human coronavirus 229E is an alphacoronavirus that causes mild respiratory disease. It is one of the viruses responsible for the common cold, but can cause more severe disease such as pneumonia, and bronchiolitis in those that are at increased risk of disease. Distribution is worldwide.

Human coronavirus OC43 is a betacoronavirus that causes mild respiratory disease very similar to disease caused by HCoV-229E. Similarly, infections can be severe in populations that are immunocompromised. Distribution is worldwide.

Severe acute respiratory syndrome associated coronavirus is a betacoronavirus that caused the outbreak of severe acute respiratory syndrome (SARS) in China in 2002 / 2003 that spread to the rest of Asia and much of the world to a lesser extent. Disease begins with mild cold-like symptoms, but progresses to flu-like disease with high fever and possible viral pneumonia development. While distribution was nearly worldwide, SARS-CoV is not present in the human population at this time.

Middle eastern respiratory syndrome associated coronavirus is a betacoronavirus that causes severe respiratory disease very similar to SARS. This virus was discovered in Saudi Arabia in 2012 and has spread to much of the Middle East. While there have been confirmed cases of travel-associated MERS-CoV infections throughout Europe and the Western Hemisphere, it does not have a presence in these areas.

Mouse hepatitis virus is a betacoronavirus common to mice that causes differing diseases depending on the tropism of the strain contracted. Disease can include a respiratory form, enteritis, encephalitis, hepatitis and may include other organ systems as well. Disease is not common in immunocompetent animals.

The effect of temperature on coronavirus survival

Gerber et al. (2014) demonstrated that porcine plasma spiked with live PEDV and heated via the spray drying process (166°C inlet and 80°C outlet temperatures) is no longer infectious when fed to 2-week old pigs. This observation is supported by Pujols et al. (2013) who demonstrated that spray drying bovine plasma spiked with PEDV to a concentration of 5.15 $\log_{10}$ TCID50/g (200°C inlet temperature and 70°C and 80°C material temperature) prevented 100% of growth on VERO cells. Reverse transcription-polymerase chain reaction (RT-PCR) of these inactivated samples were still positive, with a mean Ct = 23.3 (70°C) and 23.8 (80°C), though this was decreased from the beginning mean Ct of 13.9.

TGEV has been shown to require longer periods of heating when exposed to lower temperatures to achieve complete inactivation as determined by plaque formation on pig kidney cells (Laud et al., 1981), with loss of infectivity at 54°C occurring near 50 minutes but increasing to multiple hours at temperatures of 38°C. Additionally, inactivation times were greatly reduced when TGEV was heated in a pH 8 environment versus a neutral pH (Laud et al., 1981). Further, inactivation rates demonstrated that inactivation proceeded via two thermodynamically distinct mechanisms above and below 45°C, suggesting different targets on either side of this mark. Laude et al. (1981) suggest that RNA hydrolysis occurs below this temperature and viral protein destruction occurs above.

In the presence of liquid manure, TGEV was completely inactivated (determined via tissue culture) in 30 minutes at 55°C, in one hour at 50°C, and in 2.5 hours at 45°C (Haas et al., 1995). At 40°C, complete inactivation had not yet occurred by five hours, and required 24 hours at 35°C, 2 weeks at 20°C, and was still not completely inactivated after >8 weeks at 5°C (Haas et al., 1995).

Heating of CCV to 56°C resulted in loss of infectivity of cell culture by 60 minutes (Pratelli, 2008). At 60°C the infectivity titer was decreased by 2.6 $\log_{10}$ PFU/0.1 mL by five minutes and by >4 $\log_{10}$ PFU/0.1 mL by 15 minutes (Saknimit et al, 1988). At 65°C for five minutes, CCV was decreased by 5 $\log_{10}$ TCID50 / 50µL after only five minutes and was completely inactivated by 40 minutes (Pratelli, 2008). At 75°C, infectivity was nearly absent ($10^{1.0}$ TCID50 / 50µL) at five minutes and absent at 15 minutes (Pratelli, 2008). As with TGEV, heating inactivation was greatly enhanced when performed in an alkaline environment.

Both HCoV-229E and -OC43 were shown to survive for four or more days when suspended in various media at 37°C (Sizun and Talbot, 2000), with survival still at 50% initial virus infectivity (determined via tissue culture) for HCoV-OC43 in PBS at 6 days.

Heating SARS-CoV to 56°C for 30 minutes was sufficient to decrease virus infectivity below a level that was detectable by tissue culture (Rabenau et al., 2005a). However, the addition of 20% fetal calf serum (FCS) resulted in only 1.93 (vs. >5) $\log_{10}$ TCID50 reduction in SARS-CoV. By increasing temperature to 60°C for 30 minutes all virus was again inactivated to a limit below detection, both with and without the addition of 20% FCS (Rabenau et al., 2005a). Duan et al. (2003) demonstrated that SARS-CoV is stable for at least 120 minutes at temperatures ranging from 4°C to 37°C, but completely inactivated at 56°C for 90 minutes with reduction in the infectivity of cell culture after 60 minutes. At 67°C for 60 minutes complete inactivation occurred, with reduction in infectivity occurring after 15 minutes. At 75°C, inactivation occurred at 30 minutes, with significant reduction in infectivity after 15 minutes (Duan et al., 2003). Similar results, demonstrating a significant loss of infectivity of cell culture after a short period of time with extended periods of 30 – 60

minutes being required for complete inactivation is supported by others (Darnell et al., 2004; Darnell and Taylor, 2006; Kariwa et al., 2004).

Heat inactivation of MERS-CoV required 25 minutes at 56°C to achieve a 4 log_{10} reduction on TCID50, while 15 minutes at 65°C was more than sufficient, and no loss of infectivity was observed by 2 hours at 25°C (Leclerq et al., 2014). Inactivation (determined via plaque formation) of MHV has been demonstrated to occur at 15 minutes at 60°C and very rapidly at one minute or less when heated to 80°C (Saknimit et al., 1988).

The effect of chemical disinfectants on coronavirus survival

Exposure of TGEV (dried on a disc) to hydrogen peroxide vapor resulted in complete inactivation (determined by tissue culture) at exposure times of 16 minutes or greater (Goyal et al., 2014). Other relevant disinfectants that have been evaluated include one minute contact times of 6% sodium hypochlorite diluted at 1:100 which resulted in <1 log_{10} PFU reduction in infectivity titer, a 0.55% aldehyde product and a 1:128 dilution of a 16.75% phenol product, both of which reduced the infectivity titer by only 2.27 and 2.03 log_{10} PFU, respectively (Hulkower et al., 2011). When contact times were increased to 5 minutes; a 2% gluteraldehyde disinfectant was capable of decreasing the infectivity titer by 4.5 log_{10} TCID50, and two quaternary ammonium products: a 1:64 dilution of a 15.36% solution and a 1:200 dilution of a 24% solution were also both capable of reducing infectivity titers by 4.5 log_{10} TCID50 (Brown, 1981).

Three separate quaternary ammonium chloride disinfectants: alkyl-dimethyl-benzyl-ammonium chloride (ADMBA) (2.5mg/mL), benzalkonium chloride (BKC) (1.75 mg/mL), and didecyl-dimethyl-ammonium chloride (DDA) (5 mg/mL) were evaluated at a range of concentrations for efficacy against CCV. All were effective at eliminating virus replication in

canine cells *in vitro* at dilutions up to 1/70; but ADMBA was not effective beyond 1/70, BKC was not effective beyond 1/90, and DDA remained effective out to 1/100, where measurement was halted (Pratelli, 2007). Pratelli (2008) also demonstrated that the effectiveness of 0.002% and 0.001% gluteraldehyde disinfectant at inactivating CCV was low (<4 $\log_{10}$ TCID50 reduction in infectivity titer) even out to 3 days of contact time at 4°C. However, both concentrations were very effective when temperature was increased to 25°C and 37°C and at these temperatures increased effectiveness was seen with at the higher concentration (Pratelli, 2008).

Sodium hypochlorite contact for one minute was shown to reduce infectivity titer of HCoV-229E by ≥3 $\log_{10}$ at concentrations of 0.5% and 0.1%, but not at 0.01% (Geller et al., 2012). Gluteraldehyde 2% also effectively reduced infectivity titers at one minute of contact (Geller et al., 2012). However, in the presence of 10% bovine serum albumin or 10% whole human blood; neither a 0.2% BKC, 0.1125% Cetrimide, nor 0.15% Cetrimide (all quaternary ammonium disinfectants) were capable of effectively reducing the TCID50 titer of HCoV-OC43 even with contact times out to 10 minutes (Wood and Payne, 1997).

Gluteraldehyde at 2.5% was effective at decreasing the TCID50 titer of SARS-CoV after 5 minutes of contact (Kariwa et al., 2004), although a small amount of infectious virus was still present after 60 minutes. At concentrations of 0.5%, Rabenau et al. (2005a) demonstrated that gluteraldehyde could effectively decrease the TCID50 of SARS-CoV in 2 minutes. Gluteraldehyde at 4% for 15 minutes, 3% for 30 minutes, and 2% for 60 minutes was also effective at decreasing the TCID50 titer of SARS-CoV in the presence of 0.3% bovine serum albumin (BSA), 10% FCS, or 0.3% BSA with 0.3% sheep erythrocytes (Rabenau et al., 2005b). Rabenau et al. (2005b) also demonstrated the effectiveness of two

quaternary ammoniums at 0.5% concentration for 30 and 60 minutes at decreasing the TCID50 titer of SARS-CoV with the same concentrations of serum products listed just previously. At very low concentrations (0.025%) of gluteraldehyde, virus reduction is only moderately effective (<3 $\log_{10}$ TCID50 reduction) at temperatures of 4°C even out to 3 days, but is very effective when temperature is increased to 25°C or 37% (Darnell et al., 2004). The same study indicated that this loss in effectiveness at low temperatures was not present at higher concentrations of 0.1% gluteraldehyde. Sodium hypochlorite (5%) at 1:50 and 1:100 dilutions, and 1% Virkon S (a peroxygen) all effectively reduced the infectivity titer of SARS-CoV by >3 $\log_{10}$ TCID50, although a slightly detectable level of infectious virus remained (Lai et al., 2005).

Dellano et al. (2009) demonstrated that 0.12% parachlorometaxylenol (a phenol), 0.21% sodium hypochlorite, and 0.10% ADMBA (a quaternary ammonium) with 79% ethanol were all effective at preventing replication of MHV in mouse liver cells following a 30 second contact time on a hard, non-porous surface.

The effect of pH on coronavirus survival

Laude (1981) demonstrated that an alkaline environment (pH 8) increased the rate at which TGEV was inactivated by temperature and decreased the infective titer by 1 – 2 $\log_{10}$ PFU. This difference was more pronounced at lower temperatures 35°C and 39°C than it was at higher temperatures such as 51°C. However, no information describing the effect of pH alone on TGEV survival was found.

Canine coronavirus is much more sensitive to inactivation in alkaline conditions than acidic conditions. Pratelli (2008) demonstrated that at 25°C and 37°C for one hour CCV was stable at neutral pH (6.34 – 7.62) but completely activated at pH 11.09 – 13.2. At pH 9.98,

CCV was also completely inactivated at 37°C and considerably decreased (> 5 $\log_{10}$ decrease in TCID50) at 25°C. At 4°C, CCV was more resistant to an alkaline environment, with little effect at pH 9.98, a 3.75 – 4.25 $\log_{10}$ TCID50 reduction in infectivity at pH 11.09, and considerable decrease in infectivity (5.75 $\log_{10}$ decrease in TCID50) at pH 13.2. Extremely acidic conditions were less effective, with complete inactivation of CCV occurring at pH 2.26 and 4.38 at 37°C, but no significant decreased infectivity at more neutral pH or lower temperatures (Pratelli, 2008).

Similar properties have been described for SARS-CoV. Darnell et al. (2004) demonstrated that in one hour at 4, 25, and 37°C SARS-CoV was completely inactivated by alkaline conditions of pH 12 and 14, but stable at these temperatures from pH 5-9. Complete inactivation also occurred at pH 1 for these temperature ranges and at pH 3 for 25 and 37°C, while at 4°C infectivity titer was reduced, but complete inactivation did not occur (Darnell et al., 2004).

This greater sensitivity to alkaline pH than acidic pH is reported for other coronaviruses as well, including HCoV-29E and MHV (Geller et al., 2009).

The effect of relative humidity on coronavirus survival

Casanova et al. (2010) demonstrated that at low temperature (4°C) TGEV and MHV can survive for an extended period of at least 28 days at a wide range of relative humidity (RH) conditions (20, 50, and 80%), however survival was somewhat decreased at higher RH. Survival was determined by a reduction cytopathic effects on tissue culture and expressed as the most probable number (MPN). Similar survival was demonstrated at 20% RH at 20°C for TGEV and MHV but when RH was increased to 50%, TGEV infectivity was decreased by ~2 $\log_{10}$ MPN by 3 days and MHV infectivity was decreased by ~3 $\log_{10}$ MPN by 5 days,

with each virus being undetectable following each of those time points, respectively. When RH was further increased to 80%, TGEV infectivity decreased at a constant rate to an infectivity reduction of 3 $\log_{10}$ MPN by 14 days at which point it became undetectable, while MHV infectivity was decreased by 4 $\log_{10}$ MPN by 7 days and undetectable at 11 days. At 40°C inactivation was more rapid at all RH levels, with infectivity dropping to undetectable levels by 5 days for TGEV and MHV at 20% RH, 12 and 24 hours for TGEV and MHV at 50% RH, and 6 hours for both TGEV and MHV at 80% RH (Casanova et al., 2010).

A similar pattern was observed for TGEV and MHV inactivation in reagent water, lake water, and pasteurized settled sewage. Casanova et al., (2009) demonstrated that at 4°C, TGEV and MHV were not greatly ($\geq$ 4 $\log_{10}$ MPN) reduced in infectivity by the time sampling was ceased at 49 days, 14 days, or 35 days for reagent water, lake water, or pasteurized settled sewage, respectively. At 25°C in reagent water, infectivity titer was decreased by 4 $\log_{10}$ MPN by days 46 and 37 for TGEV and MHV, respectively. In lake water, infectivity titer was only decreased by 2.5 $\log_{10}$ MPN for TGEV and MHV when sampling ceased at 14 days. In pasteurized settled sewage, infectivity titer was decreased by >4 $\log_{10}$ MPN by days 21 and 17 for TGEV and MHV, respectively. Ijaz et al. (1985) demonstrated that HCoV-229E was fairly stable at a range of RH (30, 50, and 80% RH) at 6°C. At 20°C, survival was greatest at 50% RH, with slightly decreased infectivity at 30% RH out to 75 hours. High RH (80%) at 20°C had the greatest effect on virus inactivation, however, a lack of precision in measurement of infectivity in the Ijaz et al. (1985) study makes quantification of survival difficult.

Discussion

Whether or not a particular method is sufficiently virucidal can be difficult to determine, and is dependent on several factors, including the infectious dose of the particular virus and how that might vary by immune status and age of the host, and differing routes of transmission. Many of the studies reviewed here equate a 3 or 4 $\log_{10}$ reduction in viral infectivity on cell culture (determined by TCID50 or PFU) to virucidal efficacy as this is considered the standard in North America and Europe (Geller et al., 2012). This should be considered critically when applying this data to evaluation of inactivation methods that might be useful versus PEDV as the minimum infectious dose for PEDV is extremely low (Thomas et al., 2015a).

It appears that the use of increased temperatures is a very efficacious means to inactivate coronaviruses in general. If appropriate temperatures are used, inactivation can be accomplished in a short time period. As temperatures decrease the time required for inactivation increases greatly. Below 45°C inactivation does not occur rapidly, but by time-related heat-induced hydrolysis of RNA; above 45°C inactivation progresses by the rapid denaturing of viral proteins (Laude et al., 1981). At 56°C complete loss of infectivity requires 60 minutes, but less than 15 minutes at 75°C (Pratelli, 2008), supporting the use of higher temperatures when rapid loss of infectivity is required.

However, the effectiveness of heat at inactivating coronaviruses is complicated by other factors including the presence of protein and pH. Heat inactivation is more effective in basic conditions (pH 8), but less effective in the presence of proteins, such as serum proteins or protein components that are present in feces. These factors should be taken into consideration when determining what temperatures to use in a PEDV decontamination

process. If feces is present at the heating step – temperatures may need to be adjusted up. If alkaline conditions are present, temperatures may potentially be decreased.

Differences between studies in disinfectant concentrations, time of exposure, and temperature that exposure occurred, make it very difficult to draw definitive conclusions regarding the comparative efficacy of different disinfectants. However, several general conclusions can be drawn. In general, all classes of disinfectants described (gluteraldehydes, halogens, quaternary ammoniums, and phenols) are suitable disinfectants for coronaviruses. In general, the efficacy of disinfectants was greatly decreased at lower concentrations, lower temperatures, shorter exposure times, and in the presence of proteins. These are all constraints that are commonly encountered when trying to disinfect environments that are contaminated with PEDV. Mixing errors or economic factors may drive down disinfectant concentrations, winter conditions lead to decreased environmental temperatures, and systems that encourage high throughput (such as some trailer washout facilities) may lead to decreased contact time of disinfectants and abbreviated washing which will allow for increased organic matter contamination. Gluteraldehyde, for example, which is commonly used to disinfect swine facilities and equipment, was ineffective at 0.025% (consistent with use of commercially-available products at 1:256 dilution) when temperatures are 4°C. However by increasing temperatures to ≥25°C or increasing concentrations of disinfectant, this was overcome.

A few simple concepts should be followed to lessen these effects. Always minimize the presence of foreign material, through washing, prior to application of a disinfectant. If increased foreign material is expected to be present (such as feces), then increase the concentration of the disinfectant used or increase contact times. When applying a

disinfectant, ambient temperatures must be ≥25°C. If cold weather prohibits this, either increase the ambient temperature through the addition of heat or increase the concentration of disinfectant being used; however increasing contact times alone may not be sufficient. If disinfectant concentrations are increased, this should be done in accordance with EPA or other regulations. Proper disinfection requires a warm environment free of foreign material, and contact of an adequate concentration of disinfectant for the proper amount of time to be effective.

Coronaviruses appear to be sensitive to extremes of pH, but are relatively stable at ranges near neutral pH. This sensitivity is variable by virus, with some (TGEV and CCV) being more sensitive to alkaline. The effect of pH also appears to decrease at lower temperatures (4°C). Inactivation of coronaviruses at different pH is mediated through an irreversible conformational change that occurs to the spike protein (Darnell et al., 2004).

The spike protein is required for fusion to host cells and virus entry, however fusion and entry occurs at different pH for different coronaviruses. For example, fusion for MHV and CCV occurs at a basic pH consistent with an enteric environment (Darnell et al., 2004 and Pratelli, 2008) while SARS-CoV fusion occurs at a neutral pH (Darnell et al., 2004). This difference may explain the variability of pH sensitivity for different coronaviruses, and makes generalizations to PEDV difficult without information specific to PEDV. However, if one considers that PEDV infects enterocytes, just as MHV and CCV, it seems likely that the sensitivity of PEDV to pH will be similar to that of MHV and CCV. Therefore, one might expect PEDV to be more susceptible to alkaline conditions than acidic conditions.

While the data regarding the effect of RH on coronavirus inactivation is fairly limited, some conclusions may be drawn. Information was summarized for TGEV, MHV, and

HCoV-229E only, but there was a similar pattern of survivability among the three. It appears that the effect of RH on inactivation is limited at low temperatures (4-6°C) with virus survival of at least 28 days (MHV and TGEV). However, as temperatures are increased, differences in RH have more of an impact on virus survival with the best evidence indicating that higher RH (80%) decreases infectivity the most, with survival times of 14 days at 20°C and only 6 hours at 40°C. In conditions of complete immersion in water, survival is extended to over a month, and longest at lower temperatures (MHV and TGEV).

These results indicate that survival of coronaviruses in the environment is variable depending on the ambient conditions. Knowing how long infectious virus will be maintained in a contaminated environment may be difficult to determine and will have to be determined and monitored on a case by case basis. If a person wishes to accomplish elimination of PEDV from an environment by time alone, a warmer ambient temperature with higher RH will be more advantageous. If these types of conditions are not a reality, then longer – possibly much longer – inactivation times should be expected.

Taken together, the various properties of coronavirus inactivation by temperature, chemical disinfectants, pH, and relative humidity point toward a general strategy for effective PEDV elimination. First, any protein-containing material such as feces should be thoroughly removed from the environment. The presence of protein has been demonstrated to protect coronaviruses and reduce the effectiveness of both thermal and chemical inactivation methods. Following the elimination of foreign material, a chemical disinfectant should be applied to the contaminated environment at an appropriate concentration. This should be performed at room temperature or above, as the efficacy of disinfectants is decreased at lower temperatures. In addition to chemical disinfection, heating of the environment to a

temperatures of 65°C for 15 minutes (or higher temperatures for shorter durations) should be performed. The addition of heat alone at this temperature and time combination should be effective, and the increased temperature will further enhance the activity of the disinfectant.

These steps should form the basis of a sound coronavirus decontamination protocol and indeed are consistent with principles for trailer and barn decontamination used today for many swine pathogens. While these guidelines are drawn from the properties of multiple coronaviruses, little of this information has been determined for PEDV. For this reason, more study is needed to determine the survival properties of PEDV at different temperatures, pH, relative humidity, and against various chemical disinfectants. This information would help guide best-practices for decontamination of PEDV from swine facilities, including barns and livestock trailers.

CHAPTER 3

EVALUATION OF TIME AND TEMPERATURE SUFFICIENT TO INACTIVATE PORCINE EPIDEMIC DIARRHEA VIRUS IN SWINE FECES ON METAL SURFACES

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Summary

Objectives: To determine temperature and time applications sufficient to inactivate porcine epidemic diarrhea virus (PEDV) on a commercial livestock trailer, and practical within the constraints of current thermo-assisted drying and decontamination (TADD) capabilities in the industry.

Materials and methods: Thirty-two 4-week-old barrows were inoculated via oral gastric tube with 5 mL of either PEDV-negative feces (Neg; n = 4), untreated PEDV-positive feces (Pos; n = 4), or PEDV-positive feces subjected to 71°C for 10 minutes (71C-10M; n = 4), 63°C for 10 minutes (63C-10M; n = 4), 54°C for 10 minutes (54C-10M; n = 4), 38°C for 12 hours (38C-12H; n = 4), 20°C for 24 hours (20C-24H; n = 4), or 20°C for 7 days (20C-7D; n = 4). These pigs served as a bioassay to determine the infectivity of virus following treatment. Bioassay results were determined by reverse transcription-polymerase chain reaction on rectal swabs collected from the inoculated pigs on days 3 and 7 post inoculation.

Results: None of the pigs in the 71C-10M and 20C-7D groups became infected with PEDV. This result differed significantly from that of the Pos group ($P < .05$). Results of the other groups did not differ significantly from that of the Pos group ($P > .05$).

Implication: Heating PEDV in the presence of feces to 71°C for 10 minutes or to 20°C (room temperature) for 7 days are sufficient to inactivate the virus, preventing transmission under the conditions of this study.

Keywords: swine, porcine epidemic diarrhea virus, inactivation, temperature, thermo-assisted drying and decontamination

Introduction

Porcine epidemic diarrhea (PED) was first described in England in 1971 in growing pigs,[1] and the causative agent, porcine epidemic diarrhea virus (PEDV), was identified in 1978.[2,3] The virus spread to the rest of Europe where it caused outbreaks of diarrhea and significant losses throughout the 1970s and 1980s.[4,5] Porcine epidemic diarrhea virus is considered endemic to Europe today, but does not cause widespread significant disease. In parts of Asia, outbreaks were recognized first in 1982 and have continued to occur since.[4,5] Until recently, the virus was considered to be absent from the western hemisphere.[5,6] In May of 2013, PEDV was identified in swine in the United States for the first time. The virus has caused severe diarrhea in sows and piglets, with near 100% mortality in piglets across a wide geographical area of the United States.[6] Outbreaks of PED continue to occur in the United States, with over 6000 PEDV-positive accessions reported from 29 states as of May 2014.[7] Genetic analysis of PEDV isolates from affected farms in the United States found the virus to be 99% genetically similar to isolates from China.[8-10] Subsequent genetic analysis of PEDV isolates revealed the presence of two genetically distinct viruses in the United States.[11] Viral cluster analysis suggests both isolates originated in China, but efforts to determine the source of entry to the United States have been unsuccessful.

Although the original mode of entry of PEDV into the United States remains unknown, contaminated livestock trailers certainly represent a significant risk for movement of the virus between and within herds.[12] This is true of other swine diseases as well, including porcine reproductive and respiratory syndrome virus (PRRSV)[13] and transmissible gastroenteritis virus (TGEV)[4]. Historically, the disease risk posed by contaminated trailers has been effectively mitigated in some cases with the use of trailer washing, disinfection protocols, and thermo-assisted drying and decontamination (TADD) systems.[14] Considering the effectiveness of TADD systems to control these other diseases, and the structural similarity of PEDV to TGEV, TADD may be an efficacious means of inactivating PEDV in contaminated livestock trailers.

The objective of this study was to investigate a range of time and temperature combinations to determine if they are sufficient to inactivate PEDV in swine feces on metal surfaces similar to those found in livestock trailers.

Materials and methods

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee prior to the initiation of any experimental activity.

Source of animals and housing

Thirty-two (32) 3-week old, clinically healthy barrows were sourced from a private commercial producer in Iowa. At 72 hours after arrival, blood was collected from each pig via jugular venipuncture using a 12-mL syringe with an 18-gauge, 1.5-inch needle (Monoject; Covidien, Mansfield, Massachusetts) then transferred to an 8.5-mL plastic serum separator tube (BD Vacutainer, 8.5-mL draw; Becton, Dickinson and Company, Franklin Lakes, New Jersey). Blood was centrifuged at 2100*g* for 10 minutes and the serum portion

was split into two aliquots by pouring into two separate 5-mL snap cap tubes (BD Falcon polypropylene round-bottom tube; Becton, Dickinson and Company). One aliquot was frozen and stored at -80°C as a duplicate.

Fecal samples were collected using a commercial swab and transport system (Starswabs II; Starplex Scientific Inc, Etobicoke, Ontario, Canada). Serum and fecal samples were submitted to Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) for diagnostic testing. Pigs were negative for PEDV and TGEV (testing fecal samples) and PRRSV (testing serum samples) using virus-specific reverse transcription-polymerase chain reaction (RT-PCR) assays. All animals were positive for porcine rotavirus via PCR. Animals were PEDV-naive by serum immunofluorescent antibody testing.

On arrival, each pig was identified with a unique plastic livestock ear tag (Allflex USA, Dallas, Texas) and weighed. Following a 72-hour rest period and initial screening as described above, pigs were blocked by weight into four blocks of 8 pigs each. One pig from each block was then randomly assigned to each of eight different groups using the RAND function in Excel (Microsoft Corporation, Redmond, WA). Each group was housed in a separate room in the Iowa State University Veterinary Medical Research Institute for the duration of the study. The four pigs within each group were housed individually in elevated tubs (Figure 1). Each tub was constructed with solid dividers, completely separating pigs from one another. One of the dividers in each tub was transparent to allow each pig visual contact with one other pig. Each divided portion of the tub had dedicated water and feed sources.

Pigs were fed ad libitum an age-appropriate diet based on corn and soybean meal and free of medications. Feces fell through the plastic slatted flooring of the tub into a common

collection area below the pigs, where it fell into a holding container with water and detergent to contain feces and PEDV particles and thus reduce the potential for environmental contamination.

Study design

Combinations of time and temperature evaluated included 71°C for 10 minutes (71C-10M), 63°C for 10 minutes (63C-10M), 54°C for 10 minutes (54C-10M), 38°C for 12 hours (38C-12H), 20°C for 24 hours (20C-24H), and 20°C for 7 days (20C-7D). In addition, a positive control group (Pos) and negative control group (Neg) underwent no time interval or temperature treatment. Prior to exposure to the designated combinations of temperature and time, aluminum trays were covered with feces to replicate a contaminated livestock trailer. The Neg group utilized PEDV-negative feces; all other groups utilized PEDV-positive feces obtained as described. Treatment groups are summarized in Table 1.

The experimental unit was the individual pig. For the bioassay, the inoculum for each pig was prepared using a single aluminum tray dedicated to that pig. The tray was contaminated with feces and then exposed to the designated combinations of temperature and time.

Challenge material

Challenge material was obtained from a separate study in which 3-week-old pigs were either challenged with PEDV or left unchallenged. Forty-eight hours following challenge, when pigs were expected to be at peak virus shedding, the pigs were euthanized and feces were collected both ante and post mortem. Feces from the challenged pigs only were pooled and homogenized to ensure uniform challenge material. After pooling, PEDV-positive feces were split into 5-mL aliquots and stored in 15-mL cone-bottom centrifuge

tubes (15-mL Sterile Polypropylene Disposable Centrifuge Tube; Fisher Scientific, Pittsburgh, Pennsylvania) so that each treatment-replicate would have a dedicated sample. The samples were placed on ice until they could be frozen at -80°C approximately 1 hour later. Additional aliquots were obtained for the purpose of assessing the handling and storage process if necessary. One sample was tested via RT-PCR at the ISU VDL prior to freezing to confirm the PEDV-positive status of the feces. This sample was PEDV-positive with a cycle threshold (Ct) value of 10.5.

Feces negative for PEDV were obtained in the same way from the unchallenged pigs. Feces negative for PEDV were also split into sufficient aliquots to allow for treatment and testing needs. One aliquot of PEDV-negative feces was tested via PEDV RT-PCR at the time of collection to confirm the PEDV-negative status of the feces. This fecal sample was PEDV-negative with a reported Ct value of >40.

Time and temperature treatment

Prior to treatment, 5 mL PEDV-positive feces was applied to an aluminum tray (Figure 2) custom made to replicate a commercial hog trailer floor. Feces were spread in a thin (≤ 2 mm), even liquid layer using a disposable, flat adhesive spreader. A separate dedicated spreader was used for each tray to avoid potential cross-contamination between replicates. After application of feces, the trays were individually sampled and tested by PCR to confirm the presence or absence of PEDV RNA prior to the timed temperature treatment.

The treatment was applied to all replicates (n = 4) of a treatment group simultaneously. For treatment groups 71C-10M, 63C-10M, 54C-10M, and 38C-12H, controlled exposure to the designated combination of time and temperature was accomplished using a Fisher Scientific Isotemp Incubator (Fisher Scientific). The incubator

was pre-heated to the target temperature for each group prior to placing the trays in the incubator. The surface temperature of the trays was monitored using a Fluke model 53-2-B thermometer with a Fluke model 80PK-1 Type-K bead probe thermocouple (Fluke Corporation, Everett, Washington). Once the average temperature of the four trays had reached the target temperature, timing began.

For treatment groups 20C-24H and 20C-7D, controlled exposure to the designated combinations of time and temperature was accomplished by placing the trays in an insulated cooler that was maintained indoors at room temperature (20°C). The coolers served to insulate the trays from wide variations in temperature that might occur during the diurnal cyclic warming and cooling of the building environment. Temperatures in the coolers were monitored with a HOBO temperature data logger (Onset Computer Corporation, Bourne, Massachusetts).

Bioassay challenge

At the expiration of the assigned time, all trays were removed from the incubator or coolers, and 10 mL of sterile 0.9% sodium chloride saline (Hospira Inc, Lake Forest, Illinois) was applied to each tray to suspend the feces for ease of re-collection. Feces were sampled again to assess the presence of PEDV by PCR. The liquid slurry of feces and saline was drawn up in a 20-mL syringe that was capped and labeled with the identification number of the single pig that was to receive the mixture. Gloves were worn and changed between trays during collection to prevent cross-contamination.

Once all trays within a group had been collected, the material was taken into the respective animal rooms for inoculation of the pigs. Personnel performing the inoculation wore disposable Tyvek coveralls (DuPont, Wilmington, Delaware) and an N95 respirator

(3M, St Paul, Minnesota) that were changed between groups. Additionally, personnel wore arm-length disposable obstetrical sleeves (Agri-Pro Enterprises, Iowa Falls, Iowa) and nitrile gloves (VetOne; MWI Veterinary Supply Co, Boise, Idaho) that were changed between pigs to prevent cross contamination. After each pig had been inoculated and the obstetrical sleeves and gloves had been discarded, the Tyvek coveralls were examined for contamination. Contaminated coveralls were removed and discarded, and a new pair was donned. Inoculation was performed via gastric gavage with an 18 French rubber catheter (Kendall; Covidien, Mansfield, Massachusetts). Each pig's mouth was held open using a ¾-inch, 45° PVC elbow pipe fitting placed over the restrainer's thumb as a speculum. The catheter was then extended through the esophagus to the pig's stomach for inoculation. After administration of the challenge material and before removal of the catheter, approximately 10 mL of air was injected to clear the catheter of residual material.

After inoculation, rectal temperatures of the pigs were assessed daily using a digital rectal thermometer dedicated to each pig (VetOne; MWI Veterinary Supply Co). Diarrhea and other clinical signs were also assessed daily. On days 3 and 7 post challenge, a rectal swab was collected from each pig and tested for PEDV by RT-PCR. Tyvek coveralls, masks, gloves, and obstetrical sleeves were used when sampling pigs, employing the same procedures as when pigs were inoculated with challenge material. Pigs were not removed from their individual pens during sampling to avoid cross-contamination between individuals. Swabs from each sampling time point were immediately frozen at -80°C and submitted simultaneously to the ISU VDL to test for PEDV by N-gene-based real-time RT-PCR as previously described.[8,12]

After collection of rectal swabs on day 7 post challenge, all animals were euthanized and necropsied. Gross evaluation of all organ systems was performed and gross pathology noted. From each pig, fresh cecal and spiral colon contents, sections of fresh and 10% formalin-fixed ileum, and fresh and formalin-fixed mesenteric lymph nodes were collected. Fresh samples were immediately frozen at -80°C, and all samples were retained in the event further testing might be required to confirm the results obtained by PCR on rectal swabs.

Bioassays were considered positive if rectal swabs were PEDV-positive by RT-PCR on days 3 and 7. A Ct value of ≤ 35 was considered positive. If only one RT-PCR result was positive and the other suspect (Ct > 35 and ≤ 40) or negative, or if the pig died after day 3, formalin-fixed ileum from these individuals was submitted to the ISU VDL to test for PEDV by immunohistochemical (IHC) staining and microscopic examination. In these instances, IHC results and the presence or absence of histological lesions consistent with PEDV were used to classify the bioassay result as positive or negative.

Statistical analysis (SAS Enterprise Guide 5.1; SAS Institute, Cary, North Carolina) was performed using Fisher's exact test to evaluate differences in proportions of positive bioassays between groups with small sample sizes.

Results

All trays (28 of 28) that were covered with PEDV-positive feces (Pos; 71C-10M, 63C-10M, 54C-10M, 38C-12H, 20C-24H, 20C-7D) were PEDV-positive by RT-PCR before and after exposure to the designated combinations of time and temperature. All trays covered with PEDV-negative feces (four of four; Neg) were PEDV-negative by RT-PCR. Mean RT-PCR values of trays pre-treatment and post treatment are summarized in Table 2.

All replicates that were positive by bioassay across all groups (nine of nine) were positive by day 3, and eight remained positive through day 7. The other pig died prior to day 7.

Bioassays were PEDV-negative in 100% of the pigs (four of four) in the Neg group and in groups 71C-10M and 20C-7D. Bioassays were PEDV-positive in 25% of the pigs (one of four) in groups 63C-10M, 54C-10M, and 20C-10M. Bioassays were PEDV-positive in 50% of the pigs (two of four) in group 38C-12H and in 100% of the pigs (four of four) in the Pos group (Table 3).

A 2 × 8 Fisher's exact test of all groups simultaneously, to evaluate the overall effect of treatment on bioassay outcome, found that treatment did have a significant effect on bioassay status ($P < .05$). More specifically, bioassay outcomes for groups 71C-10M and 20C-7D were significantly different from the Pos group ($P < .05$). No other groups were significantly different from one another (Table 3).

Two animals were removed from the trial early due to illness and death unrelated to infection with PEDV. In both, removal occurred after the day-3 rectal swabs were collected, but prior to day 7. Both pigs were submitted to the ISU VDL for full necropsy and diagnostic workups to determine cause of death and PEDV status. One pig in the positive control group (Pos) was PEDV-positive on day 3 by RT-PCR on feces and was PEDV-positive by RT-PCR on feces and IHC at removal from the study. The other pig in the 71C-10M group was PEDV-negative on day 3 by RT-PCR on feces and PEDV-negative by RT-PCR on feces and IHC at removal from the study. For the pigs not removed early, across all groups, all that were positive by bioassay on day 3 remained positive on day 7 (eight of eight), and all of the pigs that were negative by bioassay on day 3 remained negative on day 7 (22 of 22),

Therefore, the bioassay outcomes, as reported in Table 3, for the two pigs removed early were considered to be sufficiently supported and were included in statistical analysis for between-group comparisons.

Discussion

The results of this study suggest that it is possible to inactivate PEDV in the presence of feces by heating trailers to 71°C for 10 minutes or by maintaining surfaces at room temperature (20°C) for at least 7 days. No other combinations of time and temperature evaluated in this study were 100% effective at inactivating PEDV.

The presence of only a single infected pig in three of the treatment groups suggests that the housing system was effective at preventing lateral transmission between pigs. This demonstrates the value of this housing model and associated biosecurity practices for further PEDV swine bioassay research.

Currently it is estimated that there are not enough livestock trailers or washing facilities in the United States to accommodate washing all livestock trailers between loads of swine (Tom Burkgren, DVM, e-mail communication, May 8, 2014). Additionally, there is a regional shortage of transporters (Jason Hocker, DVM, MS, e-mail communication, May 5, 2014), so it is difficult to shift a transporter's time from transporting swine to washing trailers while still maintaining overall hauling capacity. Washing, disinfecting, and drying times will vary among trailers, facilities, and individual protocols, but a thorough job will require a significant amount of time. A good estimate is that washing and disinfecting will require 2 hours and drying with the use of TADD will require an additional hour, for a total time investment of 3 hours (Josh Ellingson, DVM, MS, oral communication, April 29, 2014).

For farms, systems, or trucking companies that are unable to wash, disinfect, and dry trailers due to the constraints, removing the feces and bedding by scraping and subsequently heating may be practicable. The investigators do not propose that this is a preferred alternative to thoroughly washing, disinfecting, and drying trailers. Rather, this work demonstrates the value of possible alternatives, when washing, disinfection, and drying cannot be accomplished, to reduce the risk of transmitting PEDV between groups of animals. It is important to emphasize that all time measurements in this study began when the samples achieved the target temperature via direct measurement. Variations in contamination level will likely impact the amount of time it takes to achieve the target temperature.

This information may be used to prioritize significant investments in trailer decontamination facilities. If both wash and TADD facilities cannot be built simultaneously, stakeholders will have to decide which is more important. Knowing that heating trailers to 71°C for 10 minutes will inactivate PEDV in the presence of feces may suggest that priority should be given to building TADD facilities.

When washing and disinfection do occur, it is possible that small amounts of organic material may be left behind on the trailer.[15] The activity of many disinfectants is decreased in the presence of organic material.[16,17] Additionally, the physical presence of organic material may prevent disinfectant from reaching all surfaces.[17] In these instances, it is possible that infectious PEDV remains following washing and disinfection. The presence of this potentially infectious material represents a significant biosecurity risk. Inclusion of TADD into trailer decontamination protocols will help to mitigate this risk.

The complexity of trailer design may also prevent disinfectants from reaching all surfaces and all fecal contamination. Livestock trailers are not smooth-side inside, but

possess many channels, corners, hinges, and latches; all of which are capable of shielding organic matter from disinfectants. Because heat is transferred directly through metal, TADD would help mitigate this issue. However, this shielding effect of trailer design likely impacts TADD effectiveness to some degree as well. The experimental trays used in this study do not replicate this complexity and so may underestimate the risk of infection in a real-life setting.

This study used experimental group sizes of four pigs per treatment group for economic as well as facility and labor considerations. If a livestock trailer were contaminated with a small amount of infectious organic material, there is potential that many more than four animals could interact with the material and potentially become infected. For this reason, this study may underestimate the true risk of infection associated with each treatment group.

It is noteworthy that all 24 of the experimental trays that were contaminated with PEDV-positive feces and were exposed to combinations of time and temperature (71C-10M, 63C-10M, 54C-10M, 38C-12H, 20C-24H, 20C-7D) remained positive by RT-PCR following treatment. However, the bioassay results demonstrated that only five of the 24 (20.8%) contained an infectious dose of live virus, and 19 (79.2%) did not. This divergence is likely due to differences in virucidal mechanisms that result in viral destruction via membrane disruption, protein denaturation, or deterioration of genetic material.[18] Following exposure to combinations of time and temperature evaluated in this study, a sufficient amount of genetic material remained intact to interact with the primers in a RT-PCR assay. This suggests that viral inactivation occurred via membrane disruption or protein denaturation. In fact, denaturing of viral proteins can occur at higher temperatures such as those described in this study.[18] Additionally, membrane disruption can occur through desiccation of the virus, and it

was noted that feces did dry during the heating process. This illustrates that RT-PCR-positive environmental samples of trailers do not necessarily indicate infectious virus is present.

A wide range of temperatures was evaluated in this study to identify effective temperatures at the high end, and ineffective temperatures at the lower end of the range. While this was a good strategy for an initial study, it resulted in a range of temperatures each separated by 7°C or more. Many current TADD facilities operate between 63°C and 71°C.[14] Additionally, at these higher temperatures, significant fuel costs and equipment wear accompany each incremental increase in temperature. Further study evaluating a higher resolution of temperatures and time in this range is needed to optimize TADD protocols for inactivating PEDV.

Implications

- Under the conditions of this study, heating scraped, unwashed aluminum trays to 71°C for 10 minutes or allowing them to sit for 7 days at room temperature may be sufficient to prevent transmission of PEDV present in feces as determined by bioassay.
- Neither the other combinations of timed thermal treatments (63°C and 54°C for 10 minutes and 38°C for 12 hours), nor room temperature (20°C) for 24 hours are 100% effective at inactivating PEDV in feces.
- Appropriate TADD protocols may be effective at inactivating PEDV in trailers where fecal matter and bedding have been removed by scraping or when some organic matter is present following power washing and disinfection.

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Conflict of interest

None reported.

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CHAPTER 4

EVALUATION OF COMBINATIONS OF TIME AND TEMPERATURE SUFFICIENT TO INACTIVATE PORCINE EPIDEMIC DIARRHEA VIRUS (PEDV) ON A METAL SURFACE FOLLOWING A WASH WITH DETERGENT AND DISINFECTION WITH A QUATERNARY AMMONIUM CHLORIDE AND GLUTERALDEHYDE DISINFECTANT

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Introduction

Porcine epidemic diarrhea (PED) was first described in England in 1971 in growing pigs (Oldham, 1972), and the causative agent, porcine epidemic diarrhea virus (PEDV), was identified in 1978 (Pensaert and de Bouck, 1978; Chasey and Cartwright, 1978). The virus spread to the rest of Europe where it caused outbreaks of diarrhea and significant losses throughout the 1970s and 1980s (Saif et al., 2012; Pospischil et al., 2002). PEDV is considered endemic to Europe today, but does not cause widespread significant disease. In parts of Asia, outbreaks were recognized first in 1982 and have continued to occur since (Saif et al., 2012; Pospischil et al., 2002). Until recently, the virus was considered to be absent from the western hemisphere (Pospischil et al., 2002; Stevenson et al., 2013). In May of 2013, PEDV was identified in swine in the United States for the first time. The virus has caused severe diarrhea in sows and piglets, with near 100% mortality in piglets across a wide geographical area of the United States (Stevenson et al., 2013). Outbreaks of PED continue to occur in the United States, with nearly 7,000 PEDV-positive accessions reported from 30 states as of June 2014 (USDA, 2014), and 1,123 PEDV-positive premises confirmed since that data began being collected in June 2014 (USDA, 2015). Genetic analysis of PEDV

isolates from affected farms in the United States found the virus to be 99% genetically similar to isolates from China (Chen et al., 2014; Huang et al., 2013; Hoang et al., 2013). Subsequent genetic analysis of PEDV isolates revealed the presence of two genetically distinct viruses in the United States (Wang et al., 2014). Viral cluster analysis suggests both isolates originated in China, but efforts to determine the source of entry to the United States have been unsuccessful.

Although the original mode of entry of PEDV into the United States remains unknown, contaminated livestock trailers certainly represent a significant risk for movement of the virus between and within herds (Lowe et al., 2014). This is true of other swine diseases as well, including porcine reproductive and respiratory syndrome virus (PRRSV) (Dee et al., 2004) and transmissible gastroenteritis virus (TGEV) (Saif et al., 2012). Historically, the disease risk posed by contaminated trailers has been effectively mitigated in some cases with the use of trailer washing, disinfection protocols, and thermo-assisted drying and decontamination (TADD) systems (Dee et al., 2005). Further, TADD alone has been demonstrated to be capable of inactivation PEDV at a high temperature or long duration of time (Thomas et al., 2015a). However, the effective combinations identified require temperatures or times that are higher or longer than is common in many TADD facilities. Considering the effectiveness of proper trailer decontamination procedures to control other diseases, and the structural similarity of PEDV to TGEV, a comprehensive trailer decontamination procedure including a wash step, disinfection, and use of TADD should be an efficacious means of inactivating PEDV in contaminated livestock trailers.

The objective of this study was to investigate a range of industry-relevant time and temperature combinations following wash and disinfection steps to determine if they are

sufficient to inactivate PEDV in swine feces on metal surfaces similar to those found in livestock trailers.

These decontamination practices together are representative of industry standard practices for swine livestock trailer cleaning, and disinfection and so would lend insight into the usefulness of current practices at inactivating PEDV in contaminated livestock trailers and preventing its transmission between groups of pigs.

Materials and methods

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee prior to the initiation of any experimental activity.

Source of animals and housing

Thirty-six (36) 3-week old, clinically healthy barrows were sourced from a private commercial producer in Iowa. At 72 hours after arrival, blood was collected from each pig via jugular venipuncture using a 12-mL syringe with an 18-gauge, 1.5-inch needle (Monoject; Covidien, Mansfield, Massachusetts) then transferred to an 8.5-mL plastic serum separator tube (BD Vacutainer, 8.5-mL draw; Becton, Dickinson and Company, Franklin Lakes, New Jersey). Blood was centrifuged at 2100*g* for 10 minutes and the serum portion was split into two aliquots by pouring into two separate 5-mL snap cap tubes (BD Falcon polypropylene round-bottom tube; Becton, Dickinson and Company). One aliquot was frozen and stored at -70°C as a duplicate. Fecal samples were collected using a commercial swab and transport system (Starswabs II; Starplex Scientific Inc, Etobicoke, Ontario, Canada). Serum and fecal samples were submitted to Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) for diagnostic testing. All pigs were negative for PEDV and TGEV (testing fecal samples) and PRRSV (testing serum samples) using virus-specific reverse

transcription-polymerase chain reaction (RT-PCR) assays. One out of 36 (1/36) pigs were positive for porcine rotavirus via PCR, the rest were negative. All pigs were PEDV-naive by serum immunofluorescent antibody testing.

On arrival, each pig was identified with a unique plastic livestock ear tag (Allflex USA, Dallas, Texas) and weighed. Following a 72-hour rest period and initial screening as described above, pigs were blocked by weight into four blocks of 8 pigs each. One pig from each block was then randomly assigned to each of eight different groups using the RAND function in Excel (Microsoft Corporation, Redmond, WA). Each group was housed in a separate room in the Iowa State University Veterinary Medical Research Institute for the duration of the study. The four pigs within each group were housed individually in elevated tubs (Figure 3). Each tub was constructed with solid dividers, completely separating pigs from one another. One of the dividers in each tub was transparent to allow each pig visual contact with one other pig. Each divided portion of the tub had dedicated water and feed sources.

Pigs were fed ad libitum an age-appropriate diet based on corn and soybean meal and free of medications. Feces fell through the plastic slatted flooring of the tub into a common collection area below the pigs, where it fell into a holding container with water and detergent to contain feces and PEDV particles and thus reduce the potential for environmental contamination.

Study design

Treatment groups are summarized in Table 4. Combinations of time and temperature evaluated included 68°C for 10 minutes (WD-68C-10M), 66°C for 10 minutes (WD-66C-10M), 60°C for 20 minutes (WD-60C-20M), 49°C for 20 minutes (WD-49C-20M), and 20°C

for 12 hours (WD-20C-12H). In each of these groups, time and temperature combinations were evaluated following the application of a detergent, high pressure washing, and 10 minutes of contact time with a 1:256 dilution of a commercially-available quaternary ammonium chloride and gluteraldehyde combination disinfectant (Synergize, Preserve International, Reno, NV). In addition, two other treatment groups evaluated the same detergent, and high pressure wash, with 10 minutes or 60 minutes of contact time with the 1:256 Synergize (groups WD-10M and WD-60M, respectively). These groups did not undergo an additional heating step. Further, a positive control group (Pos) and negative control group (Neg) underwent no temperature treatment, disinfection, high pressure washing, or detergent use. Prior to exposure to the various decontamination procedures, aluminum trays were covered with feces to replicate a contaminated livestock trailer. The Neg group utilized PEDV-negative feces; all other groups utilized PEDV-positive feces obtained as described.

The experimental unit was the individual pig. For the bioassay, the inoculum for each pig was prepared using a single aluminum tray dedicated to that pig. The tray was contaminated with feces and then exposed to the designated decontamination procedure.

Challenge material

Challenge material was obtained from a separate study in which 3-week-old pigs were either challenged with PEDV or left unchallenged. At the conclusion of the trial, seven days following challenge, the pigs were humanely euthanized and feces were collected post mortem. The samples were frozen at -70°C until the morning of the trial when they were thawed in a cold water bath. Feces from the challenged pigs only were pooled and homogenized to ensure uniform challenge material. On the day of challenge, each challenge

dose was tested via RT-PCR at the ISU VDL. The mean cycle threshold (Ct) value of the challenge material was 22.0.

Feces negative for PEDV were obtained, stored, thawed, and tested in the same way from the unchallenged pigs. PEDV-negative feces were tested via PEDV RT-PCR at the time of collection and challenge to confirm the PEDV-negative status of the feces. These fecal samples were all PEDV-negative with a reported Ct value of >35.

Application of feces, detergent, high pressure washing, and disinfection steps for every treatment group were performed in a single room of the Iowa State University Lab Animal Resources (LAR) facilities.

Prior to any decontamination procedures, 10 mL PEDV-positive feces was applied to a 15.24 cm by 15.24 cm aluminum tray with 2.54 cm high sides, and a material thickness of 0.32 cm (Figure 4) custom made to replicate a commercial hog trailer floor. Feces were spread in a thin (≤ 2 mm), even liquid layer using a disposable, flat adhesive spreader. A separate dedicated spreader was used for each tray to avoid potential cross-contamination between replicates. Following application of feces, trays from all groups, excluding Pos and Neg, were placed in a refrigerator at 4°C for approximately 30 minutes to simulate the transport time from trailer contamination until the cleaning process would begin.

Following refrigeration, the trays were attached to a support framework constructed of PVC pipe (Figure 4) such that the trays were spaced 46 cm apart and the face containing the feces was oriented vertically. The structure was covered with disposable plastic that was replaced between each group to prevent cross-contamination. All trays were then individually sampled and tested by PCR to confirm the presence or absence of PEDV RNA prior to the application of any decontamination procedures.

Detergent and high pressure washing

The detergent and high pressure washing steps were applied to all groups except Pos and Neg. Following the sampling of contaminated trays, a detergent was applied to each tray with a Model A8019 hand-held trigger spray foamer (Ogena Solutions, LLC, Stoney Creek, ON, Canada).

Next, each tray was washed with a 1500 PSI high pressure washer (Hotsy Corporation, Englewood, CO) using cold water (Figure 4). The wand of the power washer was slowly passed over each tray in an oscillating motion for 2 seconds per tray. Immediately following the high pressure washing, all trays (excluding groups Pos, Neg, and WD-20C-12H) were swabbed for testing by PCR to confirm the presence or absence of PEDV RNA on the trays.

Disinfection

The disinfection step was applied to all groups except Pos and Neg. Following collection of the post-wash swabs, the disinfection step immediately commenced. Synergize was utilized at a dilution of 1:256 for all groups excluding Pos and Neg, which were not sham disinfected. Disinfectant was applied to each tray with a Model A8019 hand-held trigger spray foamer (Ogena Solutions, LLC, Stoney Creek, ON, Canada). Disinfectant was applied for approximately two seconds per tray to attain a uniform covering of disinfectant over the entire tray (Figure 4).

Following application, the disinfectant was left in contact with the trays for 10 minutes in groups WD-68C-10M, WD-66C-10M, WD-60C-20M, WD-49C-20M, WD-20C-12H, and WD-10M. The disinfectant was left in contact with the trays in group WD-60M for 60 minutes. At the conclusion of the assigned contact times, all trays (excluding groups Pos,

Neg, and WD-20C-12H) were swabbed for testing by PCR to confirm the presence or absence of PEDV RNA on the trays.

Time and temperature treatment

The time and temperature step was applied to all groups except Pos, Neg, WD-10M, and WD-60M. The treatment was applied to all replicates of a given treatment group simultaneously. For treatment groups WD-68C-10M, WD-66C-10M, WD-60C-20M, and WD-49C-20M, controlled exposure to the designated combination of time and temperature was accomplished using a Fisher Scientific Isotemp Incubator (Fisher Scientific) (Figure 4). The incubator was pre-heated to the target temperature for each group prior to placing the trays in the incubator. The surface temperature of the trays was monitored using a Fluke model 53-2-B thermometer with a Fluke model 80PK-1 Type-K bead probe thermocouple (Fluke Corporation, Everett, Washington). Once the mean temperature of the four trays had reached the target temperature, timing began.

For treatment group WD-20C-12H, controlled exposure to the designated combination of time and temperature was accomplished by placing the trays in an insulated cooler that was maintained indoors at room temperature (20°C). The cooler served to insulate the trays from wide variations in temperature that might occur during the diurnal cyclic warming and cooling of the building environment. Temperatures in the coolers were monitored with a HOBO temperature data logger (Onset Computer Corporation, Bourne, Massachusetts).

Bioassay challenge

At the expiration of the assigned time, all trays were removed from the incubator or cooler, and 10 mL of sterile 0.9% sodium chloride saline (Hospira Inc, Lake Forest, Illinois)

was applied to each tray to suspend the feces for ease of re-collection. Trays were sampled again to assess the presence of PEDV RNA by RT-PCR. The liquid slurry of feces and saline was collected with a 20-mL syringe (Figure 4) that was capped and labeled with the identification number of the single pig that was to receive the mixture. Gloves were worn and changed between trays during collection to prevent cross-contamination.

Once all trays within a group had been collected, the material was taken into the respective animal rooms for inoculation of the pigs. Personnel performing the inoculation wore disposable Tyvek coveralls (DuPont, Wilmington, Delaware) and an N95 respirator (3M, St Paul, Minnesota) that were changed between groups. Additionally, personnel wore arm-length disposable obstetrical sleeves (Agri-Pro Enterprises, Iowa Falls, Iowa) and nitrile gloves (VetOne; MWI Veterinary Supply Co, Boise, Idaho) that were both changed between pigs to prevent cross contamination. After each pig had been inoculated and the obstetrical sleeves and gloves had been discarded, the Tyvek coveralls were examined for contamination. Contaminated coveralls were removed and discarded, and a new pair was donned. Inoculation was performed via gastric gavage with a 14 French rubber catheter (Kendall; Covidien, Mansfield, Massachusetts). Each pig's mouth was held open using a ¾-inch, 45° PVC elbow pipe fitting placed over the restrainer's thumb as a speculum. The catheter was then extended through the esophagus to the pig's stomach for inoculation. After administration of the challenge material and before removal of the catheter, approximately 10 mL of air was injected to clear the catheter of residual material.

After inoculation, rectal temperatures of the pigs were assessed on days 3 and 7 post challenge using a digital rectal thermometer dedicated to each pig (VetOne; MWI Veterinary Supply Co). Diarrhea and other clinical signs were also assessed daily. On days 3 and 7 post

challenge, a rectal swab was collected from each pig and tested for PEDV by RT-PCR. Tyvek coveralls, masks, gloves, and obstetrical sleeves were used when sampling pigs, employing the same procedures as when pigs were inoculated with challenge material. Pigs were not removed from their individual pens during sampling to avoid cross-contamination between individuals. Swabs from each sampling time point were immediately frozen at -70°C and submitted simultaneously to the ISU VDL to test for PEDV by N-gene-based real-time RT-PCR as previously described (Chen et al., 2014).

After collection of rectal swabs on day 7 post challenge, all animals were euthanized and necropsied. Gross evaluation of all organ systems was performed and gross pathology noted. From each pig, fresh cecal and spiral colon contents, sections of fresh and 10% formalin-fixed ileum, and fresh and formalin-fixed mesenteric lymph nodes were collected. Fresh samples were immediately frozen at -70°C, and all samples were retained in the event further testing might be required to confirm the results obtained by PCR on rectal swabs. Bioassays were considered positive if rectal swabs were PEDV-positive by RT-PCR on days 3 and 7. A Ct value of ≤ 35 was considered positive by the ISU VDL. If day 3 and 7 RT-PCR results did not agree, bioassay status would be determined by submission of formalin-fixed ileum from these individuals to the ISU VDL to test for PEDV by immunohistochemical (IHC) staining and microscopic examination. In these instances, IHC results and the presence or absence of histological lesions consistent with PEDV would be used to classify the bioassay result as positive or negative.

Statistical analysis

Results of tray swabs were summarized using descriptive statistics to report mean and standard deviation (SD) of Ct values for each treatment group after each decontamination step.

Statistical analysis (SAS Enterprise Guide 5.1; SAS Institute, Cary, North Carolina) was performed using Fisher's exact test to evaluate differences in proportions of positive bioassays between groups with small sample sizes.

Results

All trays covered with PEDV-negative feces (four of four; Neg) were PEDV-negative by RT-PCR. All trays (32 of 32) that were covered with PEDV-positive feces (Pos, WD-68C-10M, WD-66C-10M, WD-60C-20M, WD-49C-20M, WD-20C-12H,WD-60M and WD-10M) were PEDV-positive by RT-PCR before any decontamination steps were conducted. Following the high pressure wash step, mean and SD Ct values for the trays that underwent this step and were sampled (groups WD-68C-10M, WD-66C-10M, WD-60C-20M, WD-49C-20M,WD-60M and WD-10M) increased to 25.1 ±2.17 from 20.3 ±0.63 for the same groups pre-wash.

Following the next step of disinfection, these same groups demonstrated a further increase in mean ±SD Ct, with observed post-disinfection Ct value of 30.1 ±2.14. Additionally, at this point one tray in group WD-68C-10M, became negative via RT-PCR with a Ct >35.

Following the final decontamination step, timed heating, mean ± SD Ct values further increased to 34.3 ±0.96 for those groups that underwent this step and were sampled (WD-68C-10M, WD-66C-10M, WD-60C-20M, WD-49C-20M, and WD-20C-12H). Additionally,

multiple more trays were RT-PCR negative at this point with Ct >35. This included one tray each from groups WD-68-10M and WD-60C-20M and two trays in group WD-66C-10M. Further, all trays in group WD-20C-12H were RT-PCR negative following this step with Ct >35.

Mean and SD RT-PCR values of trays following each decontamination step are summarized in full by group in Table 5.

Bioassays were PEDV-negative in 100% of the pigs (four of four) in the Neg group and in groups WD-68C-10M, WD-66C-10M, WD-60C-20M, WD-49C-20M, WD-20C-12H, and WD-10M. Bioassays were PEDV-positive in 100% of the pigs (four of four) in the Pos group (Table 6).

All pigs that were positive by bioassay, were RT-PCR positive by day 3 and remained positive on day 7 (four of four); and all of the pigs that were negative by bioassay, were RT-PCR negative on day 3 and remained negative on day 7 (32 of 32). Because all day 3 and day 7 RT-PCR results were in agreement, no PEDV IHC testing was performed on ileum sections and there are no IHC data to report.

A 2 × 8 Fisher's exact test of all groups simultaneously, to evaluate the overall effect of treatment on bioassay outcome, found that treatment did have a significant effect on bioassay status ($P < .05$). More specifically, bioassay outcomes for groups WD-68C-10M, WD-66C-10M, WD-60C-20M, WD-49C-20M, WD-20C-12H, and WD-10M were significantly different from the Pos group ($P < .05$) when compared in a 2 x 2 Fisher's exact test. No other groups were significantly different from one another (Table 6).

Discussion

A previous study using this bioassay model demonstrated that it is an effective model for evaluation of PEDV inactivation and infectivity (Thomas et al., 2015a). This study also demonstrated that the animal housing and handling techniques used here were capable of maintaining one infected pig within a group without passing PEDV to other pigs within the same group or to other groups.

All treatment groups were 100% effective at inactivating PEDV to the point of preventing infection in 3-week old pigs. This work clearly demonstrates the value of a complete trailer decontamination protocol that includes a wash step, disinfection step, and a final TADD step. Furthermore, it is important to note that temperatures that were found to be ineffective at inactivating PEDV under different experimental conditions (Thomas et al., 2015a) were effective at inactivating PEDV in this study - following a wash and disinfection step. These apparent differences in efficacy at the same temperature underscore the importance of thorough washing to remove organic material in any decontamination protocol.

Two groups (WD60 and WD10) that included high pressure washing and disinfection, but did not include a TADD step were also effective at inactivating PEDV under the conditions of this trial. The ability to inactivate PEDV under these conditions and failure to reliably inactivate PEDV with heating alone at several temperature ranges (Thomas et al, 2015a) indicates that whenever possible, high pressure washing and disinfection of livestock trailers should be performed.

When washing and disinfection do occur, it is possible that small amounts of organic material may be left behind on the trailer (Dee et al., 2006). The activity of many

disinfectants is decreased in the presence of organic material (ISU CFSPH, 2014; Dvorack, 2008). Additionally, the physical presence of organic material may prevent disinfectant from reaching all surfaces (Dvorack, 2008). In these instances, it is possible that infectious PEDV remains following washing and disinfection. The presence of this potentially infectious material represents a significant biosecurity risk. Inclusion of TADD into trailer decontamination protocols will help to mitigate this risk. This work serves as a guide for effective time and temperature combinations to be used following a high pressure wash and disinfection with Synergize. Specifically, 68°C for ten minutes, 66°C for 10 minutes, 60°C for 20 minutes, and 49°C for 20 minutes. Further if access to TADD facilities is not possible, storing properly washed and disinfected trailers at 20°C for 12 hours should also be expected to result in inactivation of PEDV and prevent transmission. Of course, the higher the temperature and longer the duration of heating that can be accomplished; one could assume the lower the risk would be.

The complexity of trailer design may also prevent disinfectants from reaching all surfaces and all fecal contamination. Livestock trailers are not smooth-sided inside, but possess many channels, corners, hinges, and latches; all of which are capable of shielding organic matter from disinfectants. Because heat is transferred directly through metal, TADD would help mitigate this issue. However, this shielding effect of trailer design likely impacts TADD effectiveness to some degree as well. The experimental trays used in this study do not replicate this complexity and so may underestimate the risk of infection in a real-life setting. This study used experimental group sizes of four pigs per treatment group for economic as well as facility and labor considerations. If a livestock trailer were contaminated with a small amount of infectious organic material that was not effectively removed through sanitation

practices, there is potential that many more than four animals could interact with the material and potentially become infected. For this reason, this study may underestimate the true risk of infection associated with each treatment group.

It is noteworthy that 20 out of 24 (83%) of the experimental trays that were contaminated with PEDV-positive feces and were then underwent high pressure washing with detergent with or without combinations of time and temperature in a short timeframe (WD-68C-10M, WD-66C-10M, WD-60C-20M, WD-49C-20M, WD-60M, and WD-10M) remained positive by RT-PCR following treatment. However, the bioassay results demonstrated that none of the same 24 trays (0%) contained an infectious dose of virus. This divergence is likely due to differences in virucidal mechanisms that result in viral destruction via membrane disruption, protein denaturation, or deterioration of genetic material (Laude, 1981). Following exposure to combinations of time and temperature evaluated in this study, a sufficient amount of genetic material remained intact to interact with the primers in a RT-PCR assay. This suggests that viral inactivation occurred via membrane disruption or protein denaturation. In fact, these mechanisms of viral destruction occur at temperatures higher than 45°C, such as those described in this study (Laude, 1981) Additionally, membrane disruption can occur through desiccation of the virus, which is aided by the heating process.

These same virucidal mechanisms are important for inactivation via chemical disinfection. Enveloped or “lipophilic” viruses, which include PEDV, are more sensitive to inactivation by chemical disinfection than non-enveloped or “hydrophilic” viruses (Wood and Payne, 1998; Brown, 1981) because their mechanism of action is directed towards the lipophilic viral envelope. This mechanism is independent of RNA destruction and so would inactivate the virus but leave RNA intact for propagation and detection via RT-PCR. This

further explains the disagreement between the RT-PCR results of tray swabs and the bioassay outcomes.

The heat-induced hydrolysis of RNA that can occur and result in viral inactivation is more important at temperatures below 45°C and is time-dependent (Laude, 1981). Indeed, the one group where 100% of tray swabs converted to RT-PCR negative was in the WD-20C-12H group, where exposure temperatures were below the 45°C threshold, and the time interval of the entire decontamination process was considerably longer than the other groups (12 hours). This extended timeframe likely allowed for more RNA degradation to occur through random means and resulted in the RT-PCR negatives. That is not to say, however, that the application of the disinfectant did not first render the virus non-infectious if not undetectable. Additionally, it is entirely possible that the prolonged exposure to disinfectant contributed to the RNA destruction.

These differences illustrate the important point that RT-PCR-positive environmental samples of trailers do not necessarily indicate infectious virus is present. Further, the collection of RT-PCR negative environmental samples does not necessarily indicate that infectious virus is absent (Thomas et al., 2015b). This should underscore the fact that environmental sampling to determine PEDV presence or absence may be of little value and should be employed with caution, or avoided altogether.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

Summary of PEDV inactivation

The conclusions from the literature review were consistent with the findings of the original research in this thesis (Chapters 3 and 4). From the review it was determined that coronavirus inactivation could be achieved rapidly at high temperatures, e.g. less than 15 minutes at 75°C, with increasing times at decreasing temperatures. These findings were supported by the first study (study 1), "Evaluation of time and temperature sufficient to inactivate porcine epidemic diarrhea virus in swine feces on metal surfaces." In that study, after 10 minutes PEDV was completely inactivated to the point of preventing infection in naïve pigs at 71°C. At lower temperatures for the same amount of time, inactivation was partial with 25% of pigs becoming infected following treatment temperatures of 63°C and 54°C both.

Interestingly, every tray in study 1 (24/24) was RT-PCR positive following heat treatment. This is consistent with one of the conclusions of the review that thermally mediated inactivation of coronaviruses occurs via the destruction of membrane proteins at temperatures above 45°C (Laude et al., 1981) not RNA hydrolysis. Study 2 results are also consistent with this conclusion.

However, the results of the post-treatment swabs for the 24 hour and 7 days at 20°C groups in Study 1 are difficult to explain given the previous statement. If viral inactivation proceeds via heat-induced, time-dependent hydrolysis of RNA, then one might expect these longest-time groups to have the highest RT-PCR Ct values, especially in the 7 day group.

However, these groups have the lowest Ct values, indicating the largest amount of intact RNA. The likely explanation for this is that although the process is dependent on time, it is heat-induced, so increased heat (as in the high temperature groups), in addition to denaturing protein, would result in increased RNA hydrolysis and higher Ct values.

Another conclusion form the literature review, that thermally mediated inactivation was reduced in the presence of protein, was supported by the conclusions of both study 1 and the second study, "Evaluation of combinations of time and temperature sufficient to inactivate porcine epidemic diarrhea virus (PEDV) on a metal surface following a wash with detergent and disinfection with a quaternary ammonium chloride and gluteraldehyde disinfectant" (study 2). In study 1, 71°C for 10 minutes was required for complete inactivation. However in study 2, complete inactivation occurred at every combination of time and temperature including 66°C for 10 minutes and 49°C for 20 minutes. In the second study, fecal material was first washed away thus removing the protective effect of the protein it contained. Of course, the success of these lower temperatures at inactivating the PEDV cannot be attributed solely to the removal of fecal material, as the chemical disinfectant Synergize, which contains 7% gluteraldehyde and 26% quaternary ammonium, was also used at each time and temperature combination.

In study 2 synergize was used at a dilution of 1:256, which is the equivalent of 0.03% gluteraldehyde and 0.1% quaternary ammonium. The literature review described that at these concentrations gluteraldehyde was effective at eliminating coronaviruses at 25°C, but not at 4°C – even after 3 days of contact. This is consistent with the findings of study 2, in which complete inactivation of PEDV (as determined by swine bioassay) was achieved with 0.03% gluteraldehyde after 10 minutes of contact time at 20°C. Of course the disinfectant also

contained 0.1% quaternary ammonium which certainly played a role as well. Although study 2 did not investigate this, it is reasonable to believe that had study 2 been carried out at lower temperatures, complete inactivation of PEDV may not have occurred in the disinfectant only treatment groups.

Because complete inactivation occurred in every treatment group in study 2, even those in which no heating step was undertaken, it makes it difficult to determine the role that temperature played on inactivation. So while the published data that reports that chemical disinfectants are more effective at higher temperatures (Pratelli, 2008) cannot be directly supported, it can be said that the findings of study 2 are consistent with this report.

Final conclusions and application of findings

The conclusions from the review that basic pH and higher RH are means through which coronavirus inactivation can occur were not evaluated in either study 1 or 2. While it remains plausible that these factors could be utilized for PEDV inactivation processes, the exact parameters of each must still be determined for PEDV specifically. This is especially true of pH, as it was reported that the range of pH sensitivity varies by each specific coronavirus and likely according to the optimal conditions for fusion to the host cell (Darnell et al., 2004 and Pratelli, 2008).

Much has been learned regarding the properties of coronavirus inactivation, and how they can be applied to PEDV for decontamination of swine facilities, including livestock trailers. The body of literature regarding coronavirus inactivation properties indicates that temperature, chemical disinfectants, pH, and RH can all be manipulated to achieve effective inactivation and prevention of infection. Further it was indicated that together, these methods can enhance or detract from one another. For example high heat and several chemical

disinfectants can achieve rapid inactivation of coronaviruses alone, but when combined, elevated temperatures enhance the activity of chemical disinfectants. Additionally while alkaline conditions alone can result in coronavirus inactivation in fairly short time, these conditions can also enhance the activity of temperature inactivation. And perhaps most importantly, the effectiveness of heat and chemical disinfectants are reduced by the presence of protein around the virus (such as would be the case in feces).

All of these factors should be considered together when designing a procedure for decontaminating facilities or livestock trailers of PEDV. This is supported by the effectiveness of each protocol evaluated in study 2, which incorporated several of these principles.

As summarized in study 2, the ideal livestock trailer decontamination process should include a thorough high pressure washing with detergent as its first step. The washing process serves two purposes, 1) the reduction of the amount of virus present in the trailer by removing the fecal and other organic material that contains the virus and 2) by reducing the fecal material in the trailer, the protective effects attributed to the presence of proteins will be mitigated and subsequent disinfection and heating steps will be more effective.

Following high pressure washing, the next step should be the application of a chemical disinfectant for an appropriate amount of time. Gluteraldehyde and quaternary ammonium combination products, phenols, sodium hypochlorite, peroxygen, or hydrogen peroxide products should all be expected to work. It is critical that these products be applied at the appropriate concentration, for the correct amount of time, and at temperatures of 20°C or warmer. In the case of study 2, a gluteraldehyde and quaternary ammonium product was applied at a dilution of 1:256 for 10 minutes and found to be effective. This is consistent with

product labels and with the body of literature concerning chemical disinfection of coronaviruses. If any of these parameters (time, concentration, or temperature) is decreased, then the others should be increased or effective inactivation may not occur.

Following disinfection, heating the trailer to a high temperature for a short duration of time should be performed. This will accomplish multiple goals. The increased temperature will further enhance the virucidal activity of the disinfectant as described previously. Additionally, the high temperatures alone should be sufficient to inactivate the PEDV. Study 1 indicates that a target temperature of 71°C should be reached for 10 minutes. This will accomplish inactivation of PEDV that may be remaining on the trailer embedded within feces. Study would indicate that if trailer washing and disinfection were effectively accomplished, then target temperatures could be reduced to 63°C or 54°C. It is not recommended that the lower temperature be used, as the published literature would indicate that inactivation times are prolonged at this temperature (Duan et al., 2003; Rabenau et al., 2005a; Pratelli, 2008).

If each of these steps is accomplished appropriately, complete PEDV elimination from a livestock trailer should be accomplished, thus reducing the risk for movement of the virus from positive to negative premises via contaminated trailers. Fortunately, these recommendations do not require additional steps or equipment that aren't already common to many trailer wash and decontamination facilities. In fact, these recommendations are exactly in line with current trailer decontamination protocols (Dee et al., 2005) with the exception of the increased temperature recommendations of 71°C for 10 minutes, which is certainly achievable in modern TADD facilities. This means that pork producers and stakeholders

have the tools and skills in hand to prevent PEDV spread in the US and simply must make the decision to invest in and utilize them.

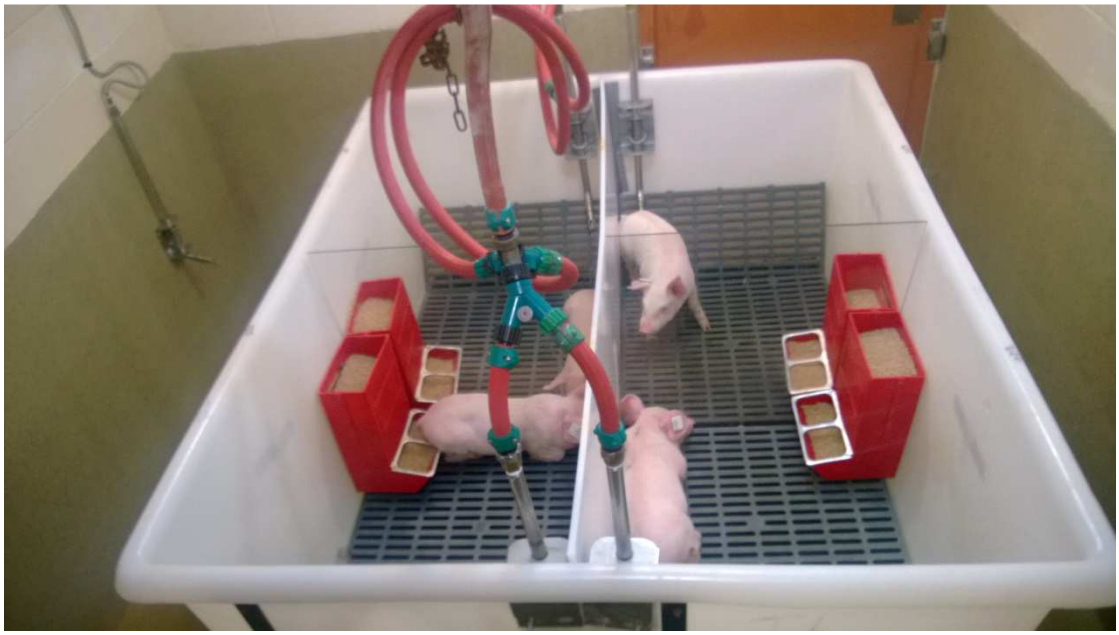

Figure 1. Elevated tubs used to house pigs for the duration of a study evaluating the ability of different combinations of time and temperature to inactivate porcine epidemic diarrhea virus (PEDV) on metal surfaces similar to those found in livestock trailers. One tub was located in each room. Each tub was split into quarters with one pig per quarter. Design of the tub prevented contact between pigs and movement of feces or other waste between tub quarters. Swine bioassays were used to determine infectivity of virus in the challenge material; and polymerase chain reaction (PCR) of day 3 and day 7 rectal swabs was used to determine bioassay status. The experimental unit was the individual pig, with four pigs per treatment group. Challenge material was prepared using a single aluminum tray, dedicated to one pig, that was contaminated with feces to replicate a contaminated livestock trailer and then exposed to the designated combinations of temperature and time. The Neg group utilized PEDV-negative feces; the other seven groups utilized PEDV-positive feces. Combinations of time and temperature evaluated included 71°C for 10 minutes (71C-10M), 63°C for 10 minutes (63C-10M), 54°C for 10 minutes (54C-10M), 38°C for 12 hours (38C-12H), 20°C for 24 hours (20C-24H), and 20°C for 7 days (20C-7D). In addition, the positive control group (Pos) and negative control group (Neg) underwent no time interval or temperature treatment. Treatment groups are described in Table 1.

Figure 2. Study described in Figure 1. Aluminum trays used to replicate trailer construction materials measured 15.24 ×15.24 cm, with 2.54-cm high sides, with a material thickness of 0.32 cm. Feces was applied to the tray (bottom left; 5 mL) and then spread in a thin layer (bottom right).

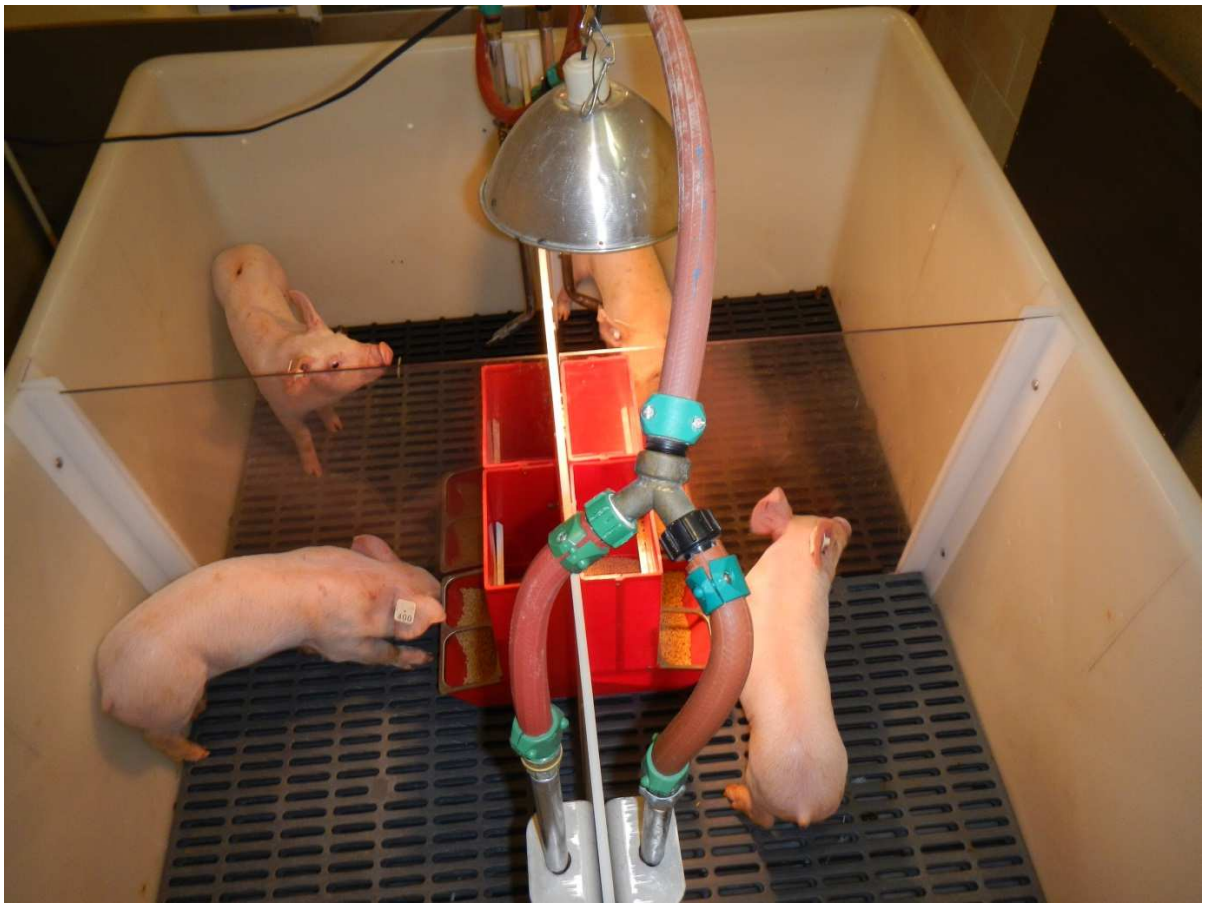

Figure 3. Elevated tubs used to house pigs for the duration of a study evaluating the ability of different combinations of time and temperature to inactivate porcine epidemic diarrhea virus (PEDV) on metal surfaces similar to those found in livestock trailers following a high pressure washing and disinfection step. One tub was located in each room. Each tub was split into quarters with one pig per quarter. Design of the tub prevented contact between pigs and movement of feces or other waste between tub quarters. Swine bioassays were used to determine infectivity of virus in the challenge material; and polymerase chain reaction (PCR) of day 3 and day 7 rectal swabs was used to determine bioassay status. The experimental unit was the individual pig, with four pigs per treatment group. Challenge material was prepared using a single aluminum tray, dedicated to one pig, that was contaminated with feces to replicate a contaminated livestock trailer and then underwent various decontamination procedures as described in Table 4. The Neg group utilized PEDV-negative feces; the other eight groups utilized PEDV-positive feces.

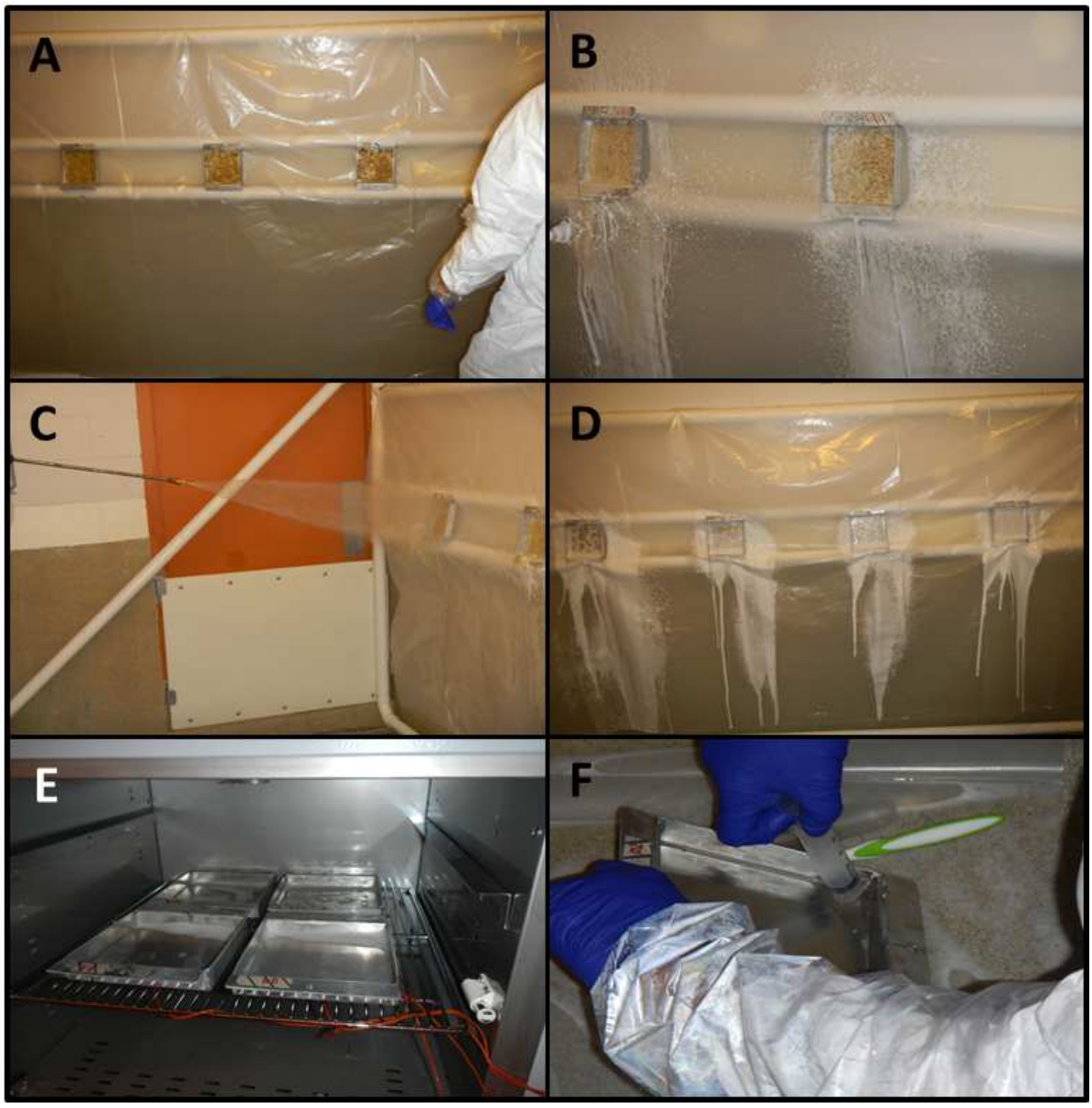

Figure 4. Study described in Figure 3. Various steps of the decontamination process. A: Aluminum trays used to replicate trailer construction materials with 10 mL of feces are attached vertically to a framework. B: Application of detergent using a hand-held spray foamer to all groups except Pos and Neg. C: High pressure washing of the trays to remove feces for all groups except Pos and Neg. D: Application of a 1:256 dilution of Synergize disinfectant for 10 minutes (6/9 groups) or 60 minutes (1/9 groups). Pos and Neg groups were not sham disinfected. E: Exposure of trays to combinations of time and temperature (5/9 groups). F: Recollection of any remaining material from trays following all decontamination steps. Material was then orally gavaged into 3 week old pigs that served as

Table 1. Time and temperature treatment groups evaluated for their ability to inactivate PEDV on metal surfaces and the field conditions they simulate*

Treatment name and description	Simulates
Neg	
No treatment, gavage of PEDV-negative feces	Negative control
Pos	
No treatment, gavage of PEDV-positive feces	Positive control
71C-10M	
Heated to 71°C in an incubator, held at 71°C for 10 minutes†	Heating via TADD‡ to a temperature of 71°C, held at 71°C for 10 minutes§
63C-10M	
Heated to 63°C in an incubator, held at 63°C for 10 minutes†	Heating via TADD‡ to a temperature of 63°C, held at 63°C for 10 minutes§
54C-10M	
Heated to 54°C in an incubator, held at 54°C for 10 minutes†	Heating via TADD‡ to a temperature of 54°C, held at 54°C for 10 minutes§
38C-12H	
Heated to 38°C in an incubator, held at 38°C for 12 hours†	Heating to 38°C for 12 hours§¶
20C-24H	
Left at 20°C for 24 hours†	Unused for 24 hours between loads of hogs, but not heated§¶
20C-7D	
Left at 20°C for 7 days†	Not heated, but unused for 1 week between loads of hogs§¶

* Study described in Figure 1.
† PEDV-positive feces placed on an aluminum tray (the challenge material) was subjected to a specified heat treatment and re-collection of feces, which were used to inoculate pigs by gavage to assess PEDV status (bioassay).
‡ Consistent with TADD protocols in some systems[14, 15]
§ Simulates pigs exposed to a PEDV-contaminated hog trailer that had undergone decontamination via the specified procedure.
¶ Temperature lower than commonly used in TADD protocols.
PEDV = porcine epidemic diarrhea virus; TADD = thermo-assisted drying and decontamination.

Table 2. Summary of results of testing pre- and post-treatment tray swabs by RT-PCR assay for PEDV*

	RT-PCR mean Ct (± SD)	
Treatment group†	**Pre-treatment**	**Post-treatment**
Neg	> 40	NA
Pos	15.22 (0.73)	NA
71C-10M	13.40 (0.30)	24.10 (0.76)
63C-10M	13.16 (0.48)	21.56 (0.71)
54C-10M	13.41 (0.32)	20.83 (0.53)
38C-12H	13.28 (1.21)	20.19 (0.09)
20C-24H	14.45 (0.57)	15.07 (0.18)
20C-7D	12.94 (0.55)	17.71 (0.41)

* Study described in Figure 1. Treatment groups and the conditions they simulate described in Table 1. Mean Ct values are summarized for swabs of trays after addition of feces, before and after exposure to increasing temperature or time of exposure.
† As Neg and Pos groups were not exposed to temperature or time treatments, no post-treatment swabs were collected for these groups.
PEDV = porcine epidemic diarrhea virus; RT-PCR = reverse transcription-polymerase chain reaction; Ct = cycle threshold.

Table 3. Summary of swine bioassay PEDV results by treatment group*

Treatment group†	Mean RT-PCR Ct values‡		PEDV-positive bioassays (%)
	Day 3 post challenge	Day 7 post challenge	
Neg	All > 40	All > 40	0/4[a] (0)
Pos	14.3, 11.4, 10.5, 15.4	18.2, 24.6, 24.4, NA§	4/4[b] (100)
71C-10M	> 40, > 40, > 40, 35.4	All > 40§	0/4[a] (0)
63C-10M	35.7, > 40, 36.2, 13.4	> 40, > 40, > 40, 16.3	1/4[ab] (25)
54C-10M	> 40, > 40, > 40, 18.8	> 40, > 40, > 40, 18.8	1/4[ab] (25)
38C-12H	> 40, > 40, 26.3, 14.1	> 40, > 40, 15.6, 18.1	2/4[ab] (50)
20C-24H	> 40, > 40, > 40, 11.5	> 40, > 40, > 40, 17.1	1/4[ab] (25)
20C-7D	All > 40	All > 40	0/4[a] (0)

* Study described in Figure1. Treatment groups described in Table 1.
† At the time of challenge, n = 4 for all treatment groups.
‡ Ct values ≤ 35 were considered positive; > 35 and ≤ 40, suspect; and > 40, negative. Day 3 and 7 swabs were used to determine bioassay status. Bioassays with inconclusive Ct values were confirmed via histopathological examination of ileum sections in conjunction with PEDV immunohistochemistry.
§ One pig in this group died prior to the end of the trial.
[ab] Values within a column with different superscripts are significantly different ($P < .05$; Fisher's exact test).
PEDV = porcine epidemic diarrhea virus; RT-PCR = reverse transcription-polymerase chain reaction; Ct = cycle threshold.

Table 4. Time and temperature treatment groups with disinfectant step evaluated for their ability to inactivate PEDV on metal surfaces and the field conditions they simulate *

Treatment name and description	Simulates
Neg	
No treatment, gavage of PEDV-negative feces	Negative control
Pos	
No treatment, gavage of PEDV-positive feces	Positive control
WD-68C-10M	
Heated to 68°C in an incubator, held at 68°C for 10 minutes†¶	Heating via TADD‡ to 68°C for 10 minutes following standard wash and disinfection§
WD-66C-10M	
Heated to 66°C in an incubator, held at 66°C for 10 minutes†¶	Heating via TADD‡ to 66°C for 10 minutes following standard wash and disinfection§
WD-60C-20M	
Heated to 60°C in an incubator, held at 60°C for 20 minutes†¶	Heating via TADD‡ to 60°C for 20 minutes following standard wash and disinfection §
WD-49C-20M	
Heated to 49°C in an incubator, held at 49°C for 20 minutes†¶	Heating via TADD‡ to 49°C for 20 minutes following standard wash and disinfection§
WD-20C-12H	
Left at 20°C for 12 hours†¶	Standard wash and disinfection, then left to dry for 12 hours without added heat§
WD-60M	
High pressure wash with detergent, 60 minutes contact with 1:256 Synergize disinfectant, no heat step†	Standard wash, increased disinfection contact time (60 minutes), with no heating or additional drying time§
WD-10M	
High pressure wash with detergent, 10 minutes contact with 1:256 Synergize disinfectant, no heat step†	Standard wash and disinfection with no heating or additional drying time§

* Study described in Figure 3.
† PEDV-positive feces placed on an aluminum tray (the challenge material) was subjected to a specified heat treatment and re-collection of feces, which were used to inoculate pigs by gavage to assess PEDV status (bioassay).
‡ Consistent with TADD protocols in some systems
§ Simulates pigs exposed to a PEDV-contaminated hog trailer that had undergone decontamination via the specified procedure.
¶ Prior to described treatment, trays were washed with detergent and a high pressure washer and disinfected with 1:256 diluted Synergize for 10 minutes.
PEDV = porcine epidemic diarrhea virus; TADD = thermo-assisted drying and decontamination.

Table 5. Summary of results of testing pre- and post-treatment tray swabs by RT-PCR assay for PEDV*

	RT-PCR mean Ct (± SD)			
Treatment group	**Pre-treatment**	**Post-wash**	**Post-disinfect**	**Post-heating**
Neg	> 35	NA	NA	NA
Pos	20.93 (0.35)	NA	NA	NA
WD-68C-10M	21.85 (0.19)	25.90 (2.09)	31.95† (3.14)	34.58† (0.39)
WD-66C-10M	21.08 (0.69)	25.98 (2.12)	30.58 (3.16)	34.30‡ (1.15)
WD-60C-20M	21.33 (0.50)	25.55 (2.09)	30.90 (1.12)	32.83† (0.45)
WD-49C-20M	21.55 (0.40)	27.55 (2.64)	31.18 (1.95)	34.20 (0.85)
WD-20C-12H	28.48 (0.74)	NA	NA	>35
WD-60M	20.68 (0.39)	26.63 (3.36)	31.65 (2.83)	NA
WD-10M	20.43 (0.25)	25.98 (1.05)	31.60 (0.66)	NA

* Study described in Figure 3. Treatment groups and the conditions they simulate described in Table 4. Mean Ct values are summarized for swabs of trays after addition of feces, following washing step, following disinfection step, and following the timed exposure to heat where. Ct values became >35 in a group, were represented as 35 for mean and SD calculation

† One swab in this group was negative with a Ct of > 35.

‡ Two swabs in this group were negative with a Ct of > 35.

NA = Not applicable - swabs of trays not collected at this point; PEDV = porcine epidemic diarrhea virus; RT-PCR = reverse transcription-polymerase chain reaction; Ct = cycle threshold.

Table 6. Summary of swine bioassay PEDV results by treatment group*

Treatment group†	Mean RT-PCR Ct values‡		PEDV-positive bioassays (%)
	Day 3 post challenge	Day 7 post challenge	
Neg	All > 35	All > 35	0/4[a] (0)
Pos	13.1, 15.0, 22.0, 12.4	18.8, 16.0, 15.5, 23.6	4/4[b] (100)
WD-68C-10M	All > 35	All > 35	0/4[a] (0)
WD-66C-10M	All > 35	All > 35	0/4[a] (0)
WD-60C-20M	All > 35	All > 35	0/4[a] (0)
WD-49C-20M	All > 35	All > 35	0/4[a] (0)
WD-20C-12H	All > 35	All > 35	0/4[a] (0)
WD-60M	All > 35	All > 35	0/4[a] (0)
WD-10M	All > 35	All > 35	0/4[a] (0)

* Study described in Figure 3. Treatment groups described in Table 4.
† n = 4 for all treatment groups.
‡ Ct values ≤ 35 were considered positive; > 35, negative. Day 3 and 7 swabs were used to determine bioassay status.
[ab] Values within a column with different superscripts are significantly different ($P < .05$; Fisher's exact test).
PEDV = porcine epidemic diarrhea virus; RT-PCR = reverse transcription-polymerase chain reaction; Ct = cycle threshold.

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